Role of non-coding RNA in the Epigenetic Inheritance of Ribosomal RNA Gene Silencing

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Summary

The nucleolus is the subnuclear body where the tandemly repeated ribosomal RNA (rRNA) genes are transcribed to give rise to ribosomal RNA, one of the major components of the ribosome. In eukaryotic cells, epigenetic mechanisms (including silent histone marks and CpG methylation) lead to a heterochromatic structure of a fraction of the rRNA genes, which is transcriptionally silent and replicates in late S-phase. In contrast, the 'active' euchromatic ribosomal DNA (rDNA) fraction replicates in early S-phase and represents rRNA genes competent for transcription whose activity is modulated according to the requirement of cell metabolism. Inheritance of silent rDNA chromatin is controlled by NoRC, the nucleolar remodeling complex comprising TIP5 and the ATPase SNF2h. NoRC binds to the rDNA promoter and represses rDNA transcription through recruitment of histone-modifying and DNA methylating enzymes. The association of NoRC with rRNA genes takes place immediately after rDNA replication and its silencing activity is modulated by the association with a non-coding RNA (pRNA). Although studies over the last decade provided insight into the chromatin organization of rRNA genes and revealed important players involved in the establishment of silent rDNA chromatin, the underlying mechanism in the inheritance as well as the structural importance of silent rDNA chromatin remained elusive.

The aims of this work were: 1) to unravel the functional role of rDNA silencing and of the NoRC complex in cell metabolism, the nucleolus and the nuclear architecture; 2) to determine the mechanism by which TIP5-pRNA association establishes and propagates the silent rDNA chromatin structure during cell division.

The results presented here revealed that depletion of TIP5 impaired rDNA silencing, upregulated rDNA transcription levels, altered the nucleolar structure, accelerated cell proliferation rates and induced cell transformation. Moreover, it is demonstrated that TIP5 mediates not only the establishment of rDNA silencing but also the formation of perinucleolar heterochromatin that contains centric and pericentric repeats. The data showed that the TIP5-mediated heterochromatin formation is indispensable for stability of silent rRNA genes and of major and minor satellite repeats.

These findings point to a role of TIP5 in protecting genome stability and suggest that it can function in the cellular transformation process.

In addition, poly(ADP-ribose) polymerase-1 (PARP1) was identified as a critical component of the NoRC complex that establishes and maintains silent rDNA chromatin. The data indicated that PARP1 associates with TIP5 and that this interaction is mediated by pRNA. Importantly, PARP1 was shown to associate with the silent rRNA genes after the passage of the replication fork, implying a role of PARP1 in the inheritance of rDNA silent chromatin during cell division. It was furthermore demonstrated that silent rDNA chromatin is a specific substrate for ADP-ribosylation and that the enzymatic activity of PARP1 is necessary to establish rDNA silencing. These results unravel a novel function of PARP1 and ADP-ribosylation in the inheritance of silent chromatin structures and thereby shed light on how epigenetic marks are transmitted during each cell cycle.

Zusammenfassung

Der Nukleolus ist der subnukleare Körper, in welchem die tandemartig hintereinander liegenden ribosomalen RNA (rRNA) Gene transkribiert werden und somit ribosomale RNA entsteht, einer der wichtigsten Komponenten des Ribosoms. In eukaryotischen Zellen führen epigenetische Mechanismen (einschließlich inaktiver Histon-Modifizierungen und CpG-Methylierung) zu heterochromatischen Strukturen eines Bruchteils der rRNA Gene. Diese Gene sind transkriptionell inaktiv und werden in der späten S-Phase repliziert. Im Gegensatz dazu werden die "aktiven" euchromatischen rRNA-Gene in der frühen S-Phase repliziert und sind für die Transkription kompetent. Die Aktivität dieser Gene wird entsprechend der Anforderung des Zellstoffwechsels moduliert. Die Etablierung inaktiver rRNA Gene wird durch den Proteinkomplex NoRC kontrolliert, welcher aus TIP5 und der ATPase SNF2h besteht. NoRC bindet an den rDNA Promotor und reprimiert die rDNA Transkription durch die Rekrutierung von Histon-modifizierenden und DNA-methylierenden Enzymen. Die Interaktion von NoRC mit den rRNA-Genen erfolgt unmittelbar nach der Replikation der rDNA und die Inaktivierung wird durch die Assoziation mit einer nicht-kodierenden RNA (pRNA) etabliert. Studien der letzten Jahre haben Einblicke in die Chromatin-Organisation der rRNA Gene gegeben und identifizierten wichtige Akteure bei der Etablierung inaktiven rDNA Chromatins. Der zu Grunde liegende Mechanismus in der Vererbung sowie die strukturelle Bedeutung der inaktiven rRNA Gene blieben jedoch unklar.

Die Ziele dieser Arbeit waren: 1) die funktionelle Rolle der rDNA Inaktivierung und des NoRC Komplex im Zellstoffwechsel, im Nukleolus sowie in der nukleären Organisation aufzudecken; 2) den Mechanismus der Etablierung und Ausbreitung inaktiver rDNA Chromatin-Struktur durch die TIP5-pRNA Assoziation zu bestimmen.

Die Resultate dieser Arbeit zeigen, dass die Deletion von TIP5 die Inaktivierung der rRNA Gene beeinträchtigt, die rDNA Transkription hochreguliert, die Struktur des Nukleolus verändert, die Zellproliferation beschleunigt und die Zelltransformation induziert. Darüber hinaus zeigen die Ergebnisse, dass TIP5 nicht nur für die Etablierung

inaktiver rDNA verantwortlich ist, sondern auch für die Bildung von perinucleolärem Heterochromatin, welches aus zentrischen und perizentrischen repetitiven Sequenzen besteht. Die Daten demonstrieren, dass die TIP5-vermittelte Bildung des Heterochromatins für die Stabilität der inaktiven rRNA Gene und der „major and minor satellite“ repetitiven Sequenzen unverzichtbar ist. Die Resultate deuten auf eine Rolle von TIP5 beim Schutz der Genomstabilität hin und lassen vermuten, dass TIP5 eine Rolle im zellulären Transformationprozess spielt.

Ein weiteres Ergebnis dieser Arbeit ist die Identifizierung der poly(ADP-ribose) polymerase-1 (PARP1) als wichtige Komponente des NoRC Komplexes, welcher für die Aufrechterhaltung des inaktiven rDNA Chromatins verantwortlich ist. Die Daten zeigen, dass PARP1 mit TIP5 assoziiert ist und dass diese Interaktion durch pRNA vermittelt wird. PARP1 interagiert mit den inaktiven rRNA-Genen nach dem Durchgang der Replikationsgabel, was daraufhin deutet, dass PARP1 eine wichtige Rolle in der Etablierung von inaktiven rRNA Genen während der Zellteilung spielt. Diese Ergebnisse demonstrieren weiter, dass inaktives rDNA Chromatin ein spezifisches Substrat für ADP-Ribosylierung ist und dass die enzymatische Aktivität von PARP1 notwendig ist, um inaktive rRNA Gene zu etablieren. Die Ergebnisse entschlüsseln eine neue Funktion von PARP1 und der ADP-Ribosylierung bei der Etablierung von inaktiven Chromatin-Strukturen und geben darüber Aufschluss wie epigenetische Markierungen während dem Zellzyklus übertragen werden.

Abbreviations

5meC	5-methylcytosine
Acetyl-CoA	acetyl coenzyme A
AD	auto-modification domain
ADP	adenosine diphosphate
ARTD1	ADP-ribosyltransferase diphtheria toxin-like 1
ATP	adenosine triphosphate
BER	base excision repair
bp	base pair
CAT	catalytic domain
ChIP	chromatin immunoprecipitation
CPE	core prompter element
DBD	DNA binding domain
DNA	deoxynucleic acid
Dnmt	DNA methyltransferase
DTT	dithiothreitol
FACS	fluorescence-activated cell sorting
GST	glutathione S-transferase
HA	hemagglutinin
HAT	histone acetyl transferase
HDAC	histone deacetylase
His	histidine
HMG	high mobility group
HMT	histone methyltransferase
HP1	heterochromatin protein 1
IGS	intergenic spacer sequence
kb	kilobases
KCl	potassium chloride
kDa	kilodalton
lincRNA	long intergenic non-coding RNA
LSD1	lysine-specific demethylase 1
mRNA	messenger ribonucleic acid
MBD	Methyl-CpG-binding domain
MOF	member of MYST family histone acetyltransferases, homolog of Drosophila MOF [*Homo sapiens*]
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
ncRNA	non-coding RNA
NER	nucleotide excision repair
NLS	nuclear localization signal
NORs	nucleolar organizing regions
NP-40	nonidet P-40
nt	nucleotide
PAR	poly(ADP-ribose)

PARP	poly(ADP-ribose) polymerase
PARylation	poly(ADP-ribosyl)ation
PMSF	phenylmethylsulfonylfluorid
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
PRC2	polycomb repressive complex 2
pre-rRNA	precursor ribosomal RNA
pRNA	promoter RNA
PTM	post-translational modification
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
SAM	*S*-Adenosyl methionine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1
Snf2h	sucrose non-fermenting protein 2 homologue
TIF-IB	transcription initiator factor-IB
TIP5	TTFI-interacting protein 5
TSS	transcription start site
TTF-I	transcription termination factor
UBF	upstream binding factor
UCE	upstream control element
WGR	tryptophan-glycine-arginine motif
ZF	zinc finger

Table of contents

1 Introduction

The human haploid genome has a size of approximately 3.2 billion nucleotide bases, which is about 2 meters in length. Since each cell contains a full copy of the genome, compartmentalization and compaction of DNA in the nucleus is the characteristic feature of eukaryotic cells. A fully extended DNA has to be compacted 100'000 times to fit within the nucleus. At the same time, various DNA regions remain accessible for interaction with regulatory, transcription and regulation factories [1].

1.1 Chromatin Structure and Histone modifications

The compaction of the DNA is controlled by structural arrangements of the DNA with associated histone and non-histone proteins, which form together the chromatin. The most abundant proteins within the chromatin are the core histones H2A, H2B, H3, H4 and the linker histone H1[2]. These histones are responsible for the packaging of eukaryotic DNA into a nucleoprotein complex termed chromatin. The nucleoprotein consists of an octamer of two copies each of four core histone, H2A, H2B, H3 and H4 that organizes 147 base pairs of DNA in a tight superhelix [3, 4]. Between these nucleosome cores is a variable length of linker DNA that is bound by the linker histone H1 [5]. Further diversifying the nucleosome core particle is a set of histone isoforms known as histone variants, which differ in their amino acid sequence relative to the major histone species. In some cases, the histone variants can alter the architecture of the histone octamer and as a consequence, of the chromatin structure [6-11] (reviewed in [12]).

The core histones are predominantly globular expect for the N-terminal tails which are unstructured and extended away from the globular nucleosome. A striking feature of these tails is the large number and different type of modified residues they posses. They are subjected to a vide variety of enzyme-catalyzed, covalent post-translational modification including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, deiminiation and proline isomerization (reviewed in [13]). Each histone tail within the nucleosome is characterized by multiple

posttranslational modifications, which leads to distinct chromatin signature and epigenetic profile. Since most of the histone modifications are reversible, this marking system represents a fundamental regulatory mechanism for chromatin function [14]. Histone modifications regulate transcription by affecting higher-order chromatin structure or by recruiting effector proteins that further modify chromatin, which influences many fundamental cellular processes [15]. Histone modifications are occurring in a variety of different combinations, of which each affect chromatin structure and gene transcription differently. The combinatorial occurrence of histone modification led to the concept of histone code [14, 15]. The histone code extends the information given by the genetic code and seems to be a fundamental regulatory mechanism that influences most chromatin-based processes.

In the early 20th century, the staining with basic dyes let to the observation of distinct chromatin regions of the same cell type recurrently stain bright and others are barely visible. The different stained regions were termed heterochromatin and euchromatin, respectively (reviewed in [16]). Euchromatin decondenses during interphase, whereas heterochromatin corresponds to regions of the genome that remain constantly condensed and brightly stained throughout the cell cycle. Heterochromatic genome regions contain few genes and occur around the centromeres and telomeres. They are associated with regular nucleosomal arrays of hypomethylated histones and transcription factor binding is limited (reviewed in [17]). On the contrary, euchromatin is gene rich, contains irregular nucleosomal array and is enriched in acetylated histones, and thereby easily accessible by transcription factors. In contrast to early replicating euchromatin [18], heterochromatin is replicated in the late S-phase of the cell cycle (reviewed in [19]).

Silent heterochromatic chromatin is characterized by DNA methylation (see **1.2**) and a specific pattern of histone modifications, like low levels of acetylation and high levels of certain methylated lysine residues. Methylation occurs on several lysine (Lys, K) residues in the N-terminal tails of Histone H3 and H4 and as well as in the globular domain of histones. Histone methylation is performed by histone methyltransferases (HMTs), which catalyze the transfer of a methyl group from *S*-adenosyl-methionine

(SAM) to a lysine or arginine residue. All known methyltransferases contain a conserved methyltransferase domain termed SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) [20, 21] and were according to structural differences in the catalytical SET domain classified in four subfamilies [22]. Lysine methylation can occur in three different states of lysine: mono-, di-, and trimethyl. These methylation states have different effects with respect to chromatin structure and transcription. For example, the methylation pattern of histone 3 lysine 9 (H3K9) is established by different HMT; G9a and GLP are mono- and di-methylases of H3K9, whereas SUV39H2 di- and tri-methylates a mono-methylated substrate. Tri-methylation of lysine 9 of histone 3 (H3K9me3) mediates chromatin recruitment of HP1, heterochromatin condensation and gene silencing [23, 24] Similarly, methylation of H3K27 and H4K20 are associated with a repressed state of chromatin, whereas expressed genes are associated with methylated H3K4, H3K36 and H3K79 [25, 26] (reviewed in [27]).

Histone acetylation is one of the best analyzed histone modification (reviewed in [28]). The balance between histone acetylation and deacetylation is realized through the action of enzymes termed histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs). HAT catalyzes the transfer of an acetyl group from acetyl-CoA to lysine residues [29], which can be reversed by HDACs. HDACs are frequently associated with other transcriptional repressor proteins. The interplay between HDACs and other proteins with repressive effects leads to transcriptional silencing in context with chromatin structure.

1.2 DNA methylation

DNA methylation is a highly conserved epigenetic modification of DNA, which has been found in prokaryotes as well as in eukaryotes. In higher eukaryotes, cytosines in CpG-dinucleotides are frequently converted to 5-methylcytosines (5meC). In the genome of vertebrates, approximately 80 % of all CpG-dinucleotides are subjected to methylation. DNA methylation is generally thought as a mark of silent, inactive chromatin and is therefore associated with negative regulation of transcription. Methylation of CpG-dinucleotides is accomplished by DNA methyltransferases (Dnmts), which catalyze the transfer of the methylgroup from *S*-adenosyl-methionine (SAM) to CpG-dinucleotides in genomic DNA [30]. Genome-wide methylation patterns are established during embryogenesis and are propagated during cell division by combined action of Dnmts. Interestingly, DNA methylation is the only epigenetic mark that is not removed by the replication fork. Consequently, it serves as a strong memory mark for inheritance of heterochromatin.

Specific DNA methylation is mediated by DNA methyltransferase Dnmt1, Dnmt3a and Dnmt3b, which can be divided into *de novo* and maintenance methyltransferases. Dnmt1 is considered to be the key methyltransferase in mammalian cells, which predominantly attaches a methyl group to hemimethylated DNA and is responsible for maintaining the DNA methylation patter during replication. Dnmt3a and Dnmt3b, which are able to methylate hemimethylated and unmethylated CpG-dinucleotides, are essential for *de novo* methylation. During embryogenesis Dnmt3a and Dnmt3b are responsible for the establishment of distinct methylation patterns and inactivation of the genes encoding DNMT3A and DNMT3B blocks *de novo* methylation in ES cells and mouse embryos. Both methyltransferases are required for normal mammalian development but have no influence on maintenance of imprinted methylation patterns [31].

DNA methylation affects gene expression directly or indirectly. Several DNA binding proteins, for example transcription factors, can only interact with unmethylated DNA [32], whereas CpG methylation abolishes the interaction with their target sequences.

This in turn leads to a lower transcription level. Alternatively, CpG methylation can be recognized by specific MBD (methyl-CpG-binding domain) proteins, which attract multiprotein complexes that can change chromatin structure from open to close.

DNA methylation is essential for the mammalian embryonic development [33]. In mouse development targeted mutation of the DNA methyltransferase gene results in embryonic lethality [34]. Moreover, stem cells nullizygous for the Dnmt1 gene showed elevated mutation rates and revealed the importance of DNA methylation for maintaining genome stability [35]. Studies have shown as well, that DNA methylation has been implicated in numerous biological processes, including transposable element silencing, genomic imprinting and X chromosome inactivation [36]. CpG methylation is generally accepted as unidirectional mechanism that is passed on to the daughter strand and will be inherited after cell division. Thus, both strands will be methylated equally.

Although DNA methylation has been viewed as a stable epigenetic mark, studies over the last decade have revealed that this modification is not as static as once thought. After fertilization, the paternal genome rapidly undergoes genome-wide active demethylation and remains for following rounds of cell division demethylated. Simultaneously, the maternal genome experiences gradual, passive demethylation. Active demethylation is an enzymatic process, which catalyzes the removal of the methyl group from the 5meC. By contrast, passive DNA demethylation refers to the loss of methyl group from 5meC when Dnmts are inhibited or absent during multiple round of cell division [36]. In the past decade, many enzymes have been proposed to carry out active DNA demethylation and growing evidence suggests that, depending on the context, this process may be achieved by multiple mechanisms. These include: enzymatic removal of the methyl group of 5meC, base excision repair (BER) through direct excision of 5meC, deamination of 5meC to T followed by BER of the T•G mismatch, nucleotide excision repair (NER), oxidative demethylation and radical *S*‑adenosylmethionine (SAM)-based demethylation [36].

1.3 Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation (PARylation) is a reversible covalent post-translational modification catalyzed by ADP-ribosyltransferases and is involved in many physiological and pathophysiological processes, including inter- and intracellular signaling, transcription, DNA repair pathways, cell cycle regulation, and mitosis, as well as necrosis and apoptosis (reviewed in [37]). The transfer of one ADP-ribose moiety from NAD^+ to a specific amino acid residue of substrate proteins by releasing nicotinamide is known as mono-ADP-ribosylation. Protein linked ADP-ribose can serve as an acceptor and elongation of ADP-ribose via ribose-ribose bonds generates linear and multiple branched chains of poly(ADP-ribose) (PAR) of different length and complexity [38, 39]. Poly-ADP-ribosylation reactions occur in multicellular eukaryotes and are synthesized by member of the poly(ADP-ribose) polymerase (PARP) family [37].

PARP1 (also known as ARTD1 [40]) is the most abundant (1 – 2 million copies per cell) nuclear chromatin associated protein [39]. PARP1 synthesizes polymers of ADP-ribose (PAR) that consist of long branched structures with up to 200 ADP-ribose units *in vitro* [41, 42]. PARP1 is composed of three functionally distinct domains: the N-terminal DNA binding domain (DBD), the central auto-modification domain (AD) and the C-terminal catalytic domain (WGR/CAT) responsible for PAR formation (**Figure 1**) [43].

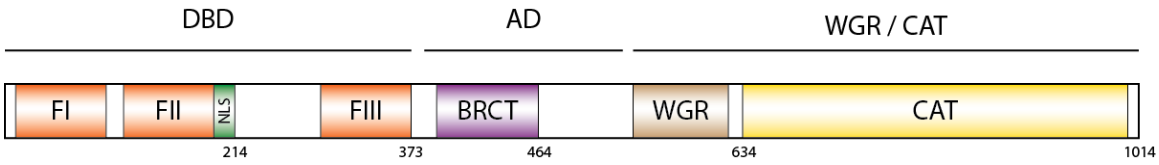

Figure 1 | Domain structure of human PARP1
DBD: DNA-binding domain; AD: Auto-modification domain; FI - FII: zinc finger I – II; FIII: zinc binding motif III; NLS: Nuclear localization signal; CAT: catalytic domain; BRCT: breast cancer 1 protein (BRCA1) C-terminus domain; WGR: tryptophane (W), glycine (G), arginine (R) rich domain. Numbers indicate amino acid positions.

The DBD contains two zinc fingers and a zinc finger binding motif: the first two were reported to bind DNA strand breaks and to be involved in protein-protein interaction [37, 44], whereas the recently discovered third zinc binding motif is essential for the catalytic

activity of PARP1 [45]. Additionally, DBD also contains a bipartite nuclear localization signal (NLS) that targets PARP1 to the nucleus [46]. The auto-modification domain is subjected to numerous post-translational modifications such as PARylation by PARP1 itself, acetylation by p300/CBP as well as sumoylation or phosphorylation [47-49]. For the C-terminal catalytic domain, at least three distinct enzymatic reactions were postulated to be required for the synthesis of free or PARP-associated linear or branched poly-ADP-ribose: the attachment of the first ADP-ribose moiety to substrate (initiation reaction), the addition of further ADP-ribose units onto the mono-(ADPribosyl)ated substrate (elongation reaction), and the generation of branching points (branching reaction) [50].

PARP1 is involved in many nuclear processes, playing key roles in DNA repair and maintenance of genomic integrity, regulation of chromatin structure and transcription. Although historically studied in the context of DNA genotoxic stress signalling, more recent studies have revealed a paradoxical dual contribution of PARP1 in regulating the composition of chromatin [50, 51]. PARP1 was implicated in the formation of chromatin structures that are permissive to transcription. In MCF-7 breast cancer cells, PARP-1 localizes to the promoters of almost all actively transcribed genes and acts to exclude linker histone H1 from a subset of PARP1-stimulated promoters [52, 53]. On the other hand, PARP1 was reported to bind to constitutive heterochromatin regions, including the centromeres [54] and telomeres [55]. In *Drosophila*, genetic studies indicated that PARP1 is necessary to organize the chromatin structure of nucleoli and heterochromatin domains and to silence retrotransposable elements [56, 57]. The enzymatic activity of PARP1 was proposed as the switch event that might distinguish between a PARP1 with co-repressor and co-activator function. The ability to disrupt chromatin structure by PARylation of histones and destabilizing nucleosomes was one of the earliest functional effects of PARP1 activity to be characterized [39, 58-60] (reviewed in [61]). The role of parylation in decondensing chromatin finds its best example in the rapid accumulation of PAR at heat shock loci in response to heat shock in *Drosophila* [62]. dPARP is required for heat shock-induced ‘‘puffing’’ (i.e., chromatin decondensation) and knockdown of dPARP or treatment with a PARP inhibitor prevents heat shock-induced nucleosome loss

and enhanced transcription at the Hsp70 gene [63]. However, examples exist where PARP1, when acting as co-activator, does not require its enzymatic activity [64-66]. Moreover, PAR activities where also found to be associated with the formation of heterochromatin. PARP1 activity was proposed to be an important determinant in telomere regulation and centrosome function in mammalian cells [54, 55]. Consistent with this, in *Drosophila*, parylated modified proteins were found particularly enriched in nucleoli and in the heterochromatic chromocenter regions [57]. From all these studies it emerged a picture where PARP1 and its associated activity regulate both euchromatic and heterochromatic regions. However the molecular mechanisms that mediate this interplay are yet to be elucidated.

1.4 Ribosome biogenesis

Ribosome biogenesis is a major cellular undertaking that occurs in the well-defined nucleolar compartment, the nucleolus, where the synthesis of ribosomal RNA (rRNA) and the assembly of ribosomes take place. Transcription of ribosomal DNA (rDNA) by Polymerase 1 (Pol I) generates rRNA precursors (pre-rRNA, 45S in mouse, 47S in human) that are subsequently cleaved and processed into three larger 28S, 18S and 5.8S rRNAs. These rRNAs are then further packed with around 80 ribosomal proteins to form the large 60S and small 40S subunits of ribosomes. Initial assembly of the ribosome occurs co-transcriptionally with 47S pre-rRNA synthesis leading to a 90S precursor particle, a process elegantly visualized in the 'Miller spread' electron micrographs [67].

Ribosome biogenesis is regulated at multiple levels, including transcription and processing of rRNA and export of the ribosome particles, and changes in this commitment are likely to have repercussions on cellular economy. For example, cells keep rRNA transcriptional activity under tight surveillance to limit excessive energy consumption for ribosome production, which could potentially deplete the cells from nutrients required for other essential processes. Control point in the complex process of ribosome biogenesis is the transcriptional regulation of rRNA genes, which requires a designated set of transcription factors and RNA polymerase I (see **1.5.2**).

Ribosomal biogenesis is not only the most complex undertaking of proliferating cells, it is also a major metabolic task. In proliferating cells, synthesis of rRNA represents the major transcriptional activity, accounting for 35 - 60 % of all cellular transcription and 80 % of total RNA content [67]. Ribosome production is associated with accurate cell growth and proliferation and it represents a huge investment of resources and energy for a cell. Cells are hardwired to respond to environmental stimuli by inducing the synthesis of protein translation machinery to coordinate cell growth and proliferation. Consistent with this, practically all signal transduction pathways activated by growth factor stimulation (including Myc and Ras) can induce ribosomal synthesis by modulating the activity of Pol I transcription factors and/or increase the rate of translational initiation [68, 69].

An important relationship exists between cell cycle and ribosome production. This balance is maintained in the cells through checkpoints, which ensures that translation of mRNA occurs at appropriate levels and during a specific window of the cell cycle. In cancer this balance can be broken, resulting in uncoupling proteins synthesis from cell growth and proliferation and in the regulation of translation (reviewed in [70]). According to this, several gene products involved in ribosomal biogenesis and protein translation are associated with tumor susceptibility (e.g. mutated ribosomal protein S19 in Diamond-Blackfan Anemia and mutated DKC1 in dyskeratosis congenital), suggesting that cancer may have defects in ribosomal control. Consistent with this, recent data suggest an active role of ribosome biogenesis in tumorigenesis. Human non-tumor lesions characterized by an up-regulation of nucleolar function were found to be associated with an increased risk of neoplastic transformation, and evidence shows that people with inherited diseases characterized by the production of abnormal ribosomes have a very high incidence of cancer [71].

1.5 Ribosomal RNA (rRNA) genes

1.5.1 Structure

The nucleolus results from the fusion of several nucleolar organizing regions (NORs), which consist of tandemly repeated ribosomal RNA genes [reviewed in 72]). In human, 200 rDNA copies exist and they are located in a non-uniform manner between the short arm and the satellite body of the five acrocentric chromosomes 13, 14, 15, 21 and 22, in a telomere-to-centromer orientation [73]. In mouse, rDNA repeats are within the centromeric regions of chromosome 12, 15, 16, 17, 18 and 19 [73-75]. The positioning of NORs on the short arms of acrocentric chromosomes was proposed to isolate rDNA units from genes transcribed by Pol II and Pol III. This isolation is further reinforced by adjacent heterochromatic repetitive sequences, such as major and minor satellites (reviewed in [76]).

Mammalian rDNA transcription units are large, comprising approximately 43 kb nts in human and 45 kb nts in mice [20, 76, 77]. rDNA clusters are characterized by multiple alternating modules of a precursor RNA sequence (pre-rRNA, approximately 13 kb) separated by long intergenic spacer sequences (IGS, approximately 30 kb) (**Figure 2**). IGS contains elements important for the regulation of rDNA transcription, such as rDNA promoter, spacer promoter, repetitive enhancer elements and transcription termination sequences.

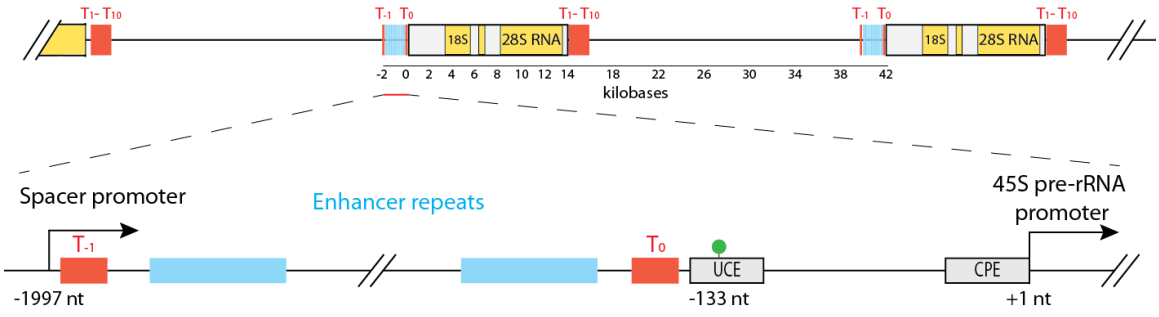

Figure 2 | Structural organization of mouse rRNA genes
The sites of transcription initiation of the 45S pre-rRNA and transcripts from the intergenic spacer promoter are indicated by arrows. -1997 nt indicates the 5' end of the intergenic spacer rRNA transcript, +1 nt the 5' end of the pre-rRNA. Terminator elements located downstream of the transcription unit (T_1–T_{10}), downstream of the spacer promoter (T_{-1}) and upstream of the gene promoter (T_0) are marked by red bars. Repetitive enhancer elements (*blue*) located between the spacer promoter and major gene promoter of the mouse gene promoter are also indicated. The green lollypop indicates the critical cytosine at position -133 (see **1.5.2**). UCE: upstream control element; CPE: core promoter element.

rRNA transcription is controlled by the Polymerase I (Pol I). The promoter from which 45S pre-RNA transcripts originate consists of two essential and specifically spaced sequences: the Pol I initiation site proximal core promoter element (CPE) and an upstream control element (UCE), which are located within a 150 bp-long DNA sequence (**Figure 2**) [78, 79]. Initiation of mammalian rDNA transcription requires the synergistic action of UBF [80] and the promoter selectively factor, TIF-IB in mouse and SL1 in human [81]. UBF contains several high mobility group (HMG) boxes, a motif known to bend DNA, and interacts with the minor groove of DNA. The tandem HMG boxes enable a single UBF dimer to loop approximately 140 bp of DNA into a single turn, thereby bringing the CPE and the UCE into close proximity by inducing a nucleosome-like structure called 'enhancosome' [82]. UBF was described to regulate rRNA gene transcription in several ways: by recruiting Pol I to the rDNA promoter, by stabilizing binding of TIF-IB/SL1, and by displacing histone H1 (**Figure 3**) [83, 84].

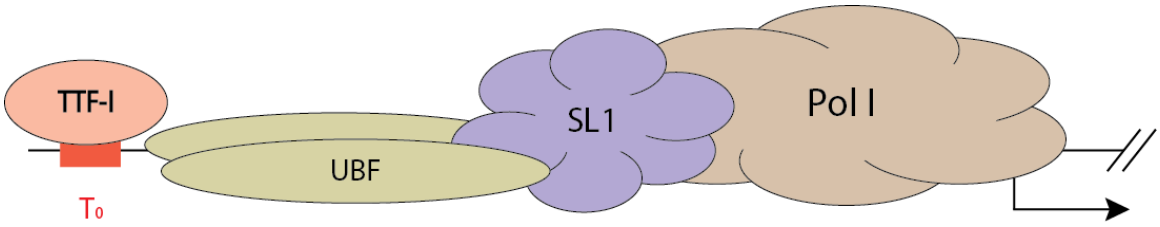

Figure 3 | Basal factors required for transcription initiation
The ellipsoids show the factors that are associated with the rDNA promoter and Pol I, respectively. TTF-I is associated with the upstream terminator T_0. Synergistic binding of UBF and TIF-IB/SL1 to the rDNA promoter is required for the recruitment of RNA polymerase I (Pol I) and multiple Pol I-associated factors to the transcription start site to initiate pre-rRNA synthesis.

In rats, mice, *Drosophila* and *Xenopus*, the IGS region contains one or more Pol I promoters (spacer promoters) that share sequence homology to the core region of the main rDNA promoter [85-88]. Transcripts originating from spacer promoters are co-directional with pre-rRNA synthesis and have been shown to enhance transcription activity of the main rDNA promoter, possibly by delivering Pol I moieties [86, 89]. Recently, intergenic spacer rRNA transcripts (IGS rRNA) were shown to have a crucial function in rDNA silencing. In mice, intergenic transcripts originating from the spacer promoter that locates approximately 2 kb upstream from the pre-rRNA start site are processed into a heterogeneous population of 200–250 nucleotide RNAs, dubbed

promoter RNA (pRNA) as their sequence matches the rDNA promoter [90]. The pRNA associates with nucleolar remodeling complex (NoRC, described in **1.5.3**), thereby maintaining silencing of rDNA chromatin [91]. As the steady-state level of IGS rRNA is very low, several models were proposed for the mechanisms accounting for the under-representation of IGS transcripts: the spacer promoter could be extremely weak, IGS transcripts could be rapidly degraded, the synthesis of IGS rRNA could be restricted to a small fraction of rDNA repeats, or IGS rRNA could be synthesized during a defined time during the cell cycle. Recent data showed that IGS rRNA synthesis is achieved by a specific class of active rRNA genes during a restricted time window of S phase (early) [91]. As synthesis and processing of IGS RNA transcripts generate pRNA (as described in **1.6**), which is a key player for establishment of silent rDNA chromatin, the data suggests that cells carefully tune the timing of IGS rRNA transcription/processing for the establishment and propagation of rDNA heterochromatin through cell division (see **1.6**).

rDNA transcription units are flanked at their 5' and 3' ends by one or more terminator elements that are recognized by transcription termination factors (TTF-I) (**Figure 2**). TTF-I is a multifunctional protein that binds to specific terminator elements (T_1 - T_{10}) downstream of the transcription unit and mediates transcription termination and replication fork arrest [92]. A similar sequence element, defined as T_0 is located immediately upstream of the ribosomal gene promoter. The conservation of a binding site for a Pol I transcription terminator protein adjacent to the gene promoter suggested that TTF-I may also exert some essential function in transcription initiation. Indeed, binding of TTF-I to the promoter-proximal terminator stimulates *in vitro* transcription of chromatinized rDNA templates by affecting rDNA nucleosomal positioning and occupancy at the rDNA promoter [93, 94].

Involvement of TTF-I in a structure mediating interaction between the main gene promoter and the 3'-rDNA region has also been recently proposed [95]. The involvement of TTF-I in forming the spacer-main gene promoter loop not only suggests that IGS rRNA synthesis might not be required to enhance transcriptional activity at the main rDNA promoter, but that it might not occur at all. The major obstacle that Pol I would encounter in transcribing IGS rDNA is TTF-I that, if bound to T_0 and T_{-1} elements, might

prematurely terminate IGS rRNA transcripts. Thus, when IGS rRNA is synthesized, TTF-I should not be bound to either T_0 and/or T_{-1} elements. As binding of TTF-I to T_0 is a prerequisite for 45S pre-rRNA synthesis [96], it is unlikely that transcription from spacer promoter enhances the strength of the main gene promoter in the absence of TTF-I. Whether and how binding of TTF-I to T_0 and T_{-1} is abrogated during synthesis of IGS rRNA in early S-phase remains an issue to be investigated. Taken together, all these observations suggest that the dual role of spacer promoter in regulating rRNA transcription can be distinguished by its capacity either to form a loop or to drive IGS rRNA synthesis: in the first case, it stimulates pre-rRNA synthesis; in the second case, it is required for NoRC-mediated rDNA silencing [97].

1.5.2 Epigenetic features of rDNA

Despite the high levels of rRNA synthesis, not all rRNA genes are competent for transcription and loaded with RNA polymerases I. In mouse, approximately 50 % of the rRNA genes remain transcriptionally silent, even under conditions requiring a high demand for ribosomes production [98, 99]. The proportion of actively transcribed rRNA genes was identified by their susceptibility to DNA cross-linking agent psoralen, an intercalating drug that can introduce crosslinks into DNA sites that are not protected by nucleosomes [100, 101]. Using this method, in a variety of organisms the presence of two distinct classes of rRNA genes, which share distinct chromatin features, was demonstrated. Active rRNA genes are free of regulatory spaced nucleosomes and are associated with nascent pre-rRNA, whereas silent gene copies are inaccessible to the psoralen cross-linking, displaying regularly spaced nucleosomes not associated with transcription factor and Pol I [100]. In mammals, the relative amount of active and silent rRNA genes is similar in both growing and resting cells as well as during interphase and metaphase, indicating that these two chromatin structures are maintained independently of transcription activity during cell cycle [100]. Consistent with this, studies from yeast demonstrated that rRNA synthesis is determined by the summed RNA polymerase I loading rate rather than by the number of genes competent for transcription (active genes) [102].

The fact that active and silent rRNA genes are stably propagated through cell cycle, suggests that epigenetic regulatory mechanisms might be involved in the inheritance of rDNA chromatin structures. In support of this, it was demonstrated that active and silent rRNA genes are characterized by distinct epigenetic marks (**Figure 4**) [103-105].

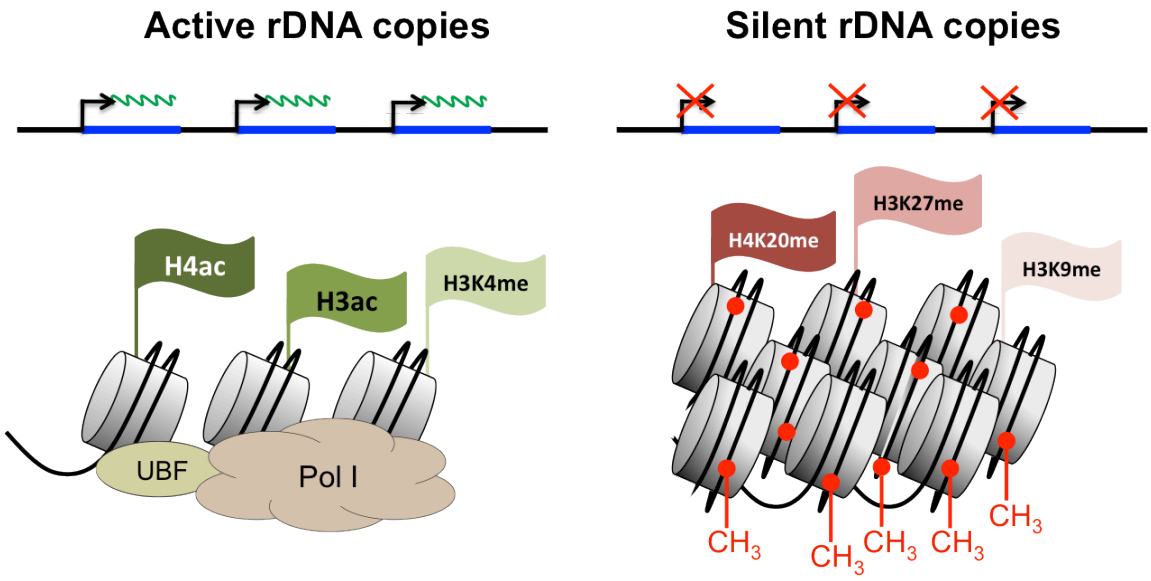

Figure 4 | rDNA repeats exist in two distinct chromatin states
Active rRNA genes posses a typical euchromatic structure consisting of acetylated histones, methylated H3K4 and hypomethylated CpG residues, and associates with UBF and Pol I transcription machinery. In contrast, silent rRNA genes are packed into heterochromatic structure containing methylated H3K9, H3K27 and H4K20, as well as hypermethylated CpG residues.

It was shown that CpG methylation was enriched in the rDNA fraction inaccessible to psoralen (silent genes) and absent in psoralen-accessible rDNA units (active genes) [106]. The relation of rDNA promoter methylation and transcription was initially strengthened by the stimulation of rRNA transcription in cell treated with 5-aza-2'-deoxycytidine (AZA), a nucleotide analogue that impairs DNA methylation process [103]. *In* vitro studies determined that CpG methylation did not impair transcription on naked rDNA templates. However, when associated into chromatin, methylated rDNA templates were not transcribed, underscoring a mechanistic link between DNA methylation and rDNA chromatin [103]. In mouse, the methylation of a single CpG within the UCE of the rDNA promoter located at position -133 (see **Figure 2**) was shown to be sufficient to impair

binding of Pol I transcription factor UBF to nucleosomal rDNA, thereby preventing initiation complex formation. Impairment of UBF binding to methylated rDNA promoter was specific to rRNA genes assembled in chromatin but not to naked rDNA templates. This finding suggested that cytosine at position -133 is exposed on the surface of the positioned nucleosome and that the addition of methyl group may represent an unfavourable sterical hindrance for UBF binding [76, 103]. In human cells, CpG methylation at positions -60 and -68 seems to act similarly (Santoro, personal communication). The correlation between methylation of the rDNA promoter and transcriptional silencing of rDNA was further supported by studies on tumors [107]. In cancer cells, rDNA transcription is enhanced, contributing to increased production of ribosomes and protein synthesis of the rapidly proliferating tumors [70] (reviewed in [108]). A lower content of rDNA methylation was reported for several tumors [109-111], strengthening the notion of the role of CpG methylation in repressing rDNA transcription [103]. Moreover, rDNA methylation levels were found to be higher in ovarian cancer patients with long progression survival as compared with that in patients with short survival, an indication that rDNA silencing levels may influence cell growth properties essential for active tumor proliferation and tumor aggressiveness [107].

The Chromatin Immunoprecipitation (ChIP) technique combined with CpG methylation measurement (ChIP-chop) provided valuable insights into the association of modified histones with either active or silent rRNA genes. Several studies revealed that mouse and human promoters of active rDNA were associated with Pol I transcription factors as well as with active histone marks, like acetylated histone H4 (H4Ac) and histone H3 dimethylated at lysine 4 (H43K4me2). In contrast, silent rRNA genes were associated with histones containing repressive marks, like H3K9me2 and H4K20me3, and with the heterochromatin protein 1 (HP1) [103-105]. Thus, active and silent rRNA genes are demarcated both by their pattern of DNA methylation and by specific modifications of their associated histones, a finding that links the 'histone code' to the 'cytosine methylation code' [104].

Additionally to these epigenetic marks, nucleosome positioning was recently proposed as an additional characteristic feature of active and silent rDNA chromatin. In

mouse, two distinct nucleosome positions at the promoter of active and silent mouse rRNA genes were identified [112]. At mouse active genes, a nucleosome occupies sequences from -157 to -2, whereas at silent genes the nucleosome covers sequences from -132 to +22. The positioning of a nucleosome over the promoter region of silent genes was found to be mediated by the nucleolar remodeling complex NoRC, whose function will be described in **1.5.3**. The specific nucleosomal architecture of active genes was proposed to bring the upstream control element (UCE) and the core promoter element (CPE) into close proximity and to facilitate the specific interactions between TIF-IB/SL1 and UBF. In this scenario, the nucleosome positioned at the rDNA promoter may provide the correct scaffolding for productive interactions between TIF-IB/ SL1 and UBF bound at the two recognition sites, which are separated by 120 base pairs. As described in **1.5.1**, a similar structure might also be driven by UBF itself that dimerizes and, after binding to DNA, has the ability to induce formation of an 'enhancesome', in which ~140 bp of DNA is organized in a 360° turn as a result of six in-phase bends generated by three of the six HMG boxes in each UBF monomer [82].

Noteworthy, the position of the nucleosome at silent rDNA promoter would locate the critical CpG dinucleotide at -133 at its 5' boundary [103]. In this position, not hindered by a nucleosome, the CpG-133 would be exposed to methylation mediated by Dnmts associated with NoRC (described in **1.5.3**) [104]. In support of this, impairment of nucleosome remodeling activity of NoRC abrogates transcriptional repression and CpG methylation of an rDNA reporter gene [105].

1.5.3 NoRC establishes rDNA silencing

The identification of the nucleolar remodeling complex NoRC, allowed to gain insights into the mechanisms that establish and propagate silent rDNA chromatin [103, 104, 113]. NoRC is composed of two subunits, the ATPase SNF2h and a 205 kDa protein termed TIP5 (TTF-I interacting protein 5), and is the key determinant that maintains individual rDNA repeats in a heterochromatic and silent state [104, 114, 115]. TIP5, the largest subunit of NoRC, shares a number of important domains with other subunits of

known human remodeling complexes like ACF, WCRF, CHRAC and WICH [116-120]. Such shared domains include a bromodomain, a PHD (plant homeodomain) finger, WAKZ motifs, a BAZ1 and a BAZ2 motif as well as AT-hooks (**Figure 5)**.

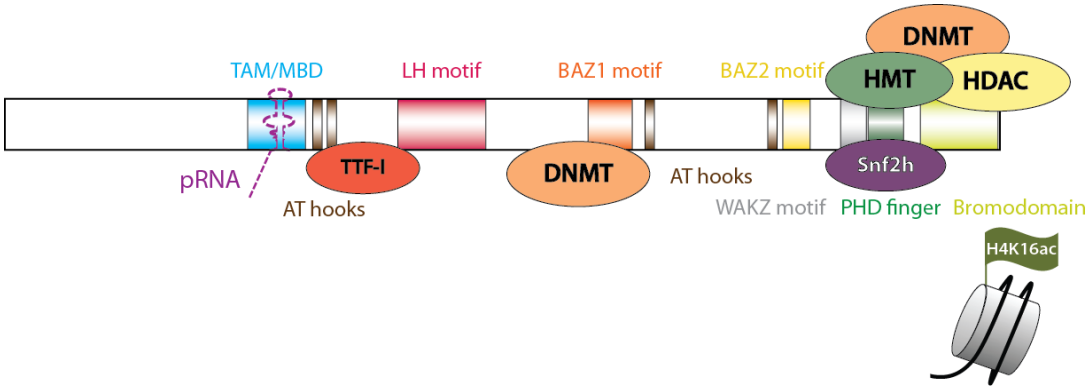

Figure 5 | Modular organization and domain structure of TIP5
Scheme illustrating the modular organization and localization of sequence motifs of TIP5. The domains of TIP5 which interact with proteins involved in the epigenetic control of rRNA genes are illustrated. The C-terminal part of TIP5 contains a PHD (plant homeodomain) finger that interacts with SNF2h and with histone methyltransferases (HMTs) and a bromodomain that interacts with histone deacetylases (HDAC1 and -2) and with histone H4 acetylated at lysine 16 (H4K16ac). DNA methyltransferases (DNMTs) interact with both the internal and the C-terminal part of TIP5. The MBD (methyl-CpG binding domain)-like TAM (TIP5/ARBD/MBD) domain is required for association with small intergenic transcripts (pRNA) that are required for NoRC-mediated heterochromatin formation.

Initial studies showed that NoRC-mediated rDNA transcriptional repression did not occur in the presence of DNA methylation and histone deacetylase inhibitors, implicating that NoRC acts by inducing DNA methylation and histone deacetylation at rDNA. NoRC was shown to associate with rDNA promoter *via* TTF-I and to repress rRNA transcription through recruitment of histone-modifying and DNA modifying enzymes (i.e. HDAC1, SETDB1, SIRT1, MOF, Dnmts), thereby establishing silent rDNA chromatin structures [104, 105, 114, 121] . Taken together, these results indicate that NoRC coordinates epigenetic events at rDNA and histones that lead to transcriptional silencing and heterochromatin formation at the rDNA locus.

Recently, the role of non-coding RNAs was recognized as an important player in controlling multiple epigenetic phenomena [122]. Non-coding RNA originating either from processed introns or intergenic DNA regions appear to be involved in the regulation of gene transcription. Recent results demonstrated that rDNA heterochromatin formation

requires the association of NoRC with a non-coding RNA (pRNA), a processed IGS RNA transcripts of the size of about 200-250 nt whose synthesis is driven by the spacer promoter (see **1.5.2**) [90]. pRNA sequence is complementary to the rDNA promoter and folds into a conserved stem–loop structure that mediates the association with the TAM domain of TIP5. Binding of TIP5 to pRNA is required for TIP5 nucleolar localization, binding to rDNA and establishment of silent rDNA chromatin [90, 123].

1.6 Inheritance of silent rDNA chromatin

During replication, chromatin marks are *bone fide* lost and have to be re-established on the newly replicated daughter strand in order to ensure cell identity. In eukaryotic organisms, chromosomal DNA replication is initiated at multiple sites on the chromosome at different times, following a temporal replication program [124, 125]. In higher eukaryotes, there is some correlation between replication timing and transcriptional activity: active genes tend to be replicated early, whereas silent and heterochromatic domains are replicated late [126]. The 'window of opportunity' model provides one of the most interesting suggestions for explaining the need for replication timing [126]. According to this model, an active gene that replicates in early S-phase is exposed to factors that are required for the formation of active transcription complexes, whereas a silent gene replicating in late S-experiences a different nuclear environment, which is more conducive for the generation of repressive structures. Consistent with this, in mouse and human cells, rRNA genes are replicated in a biphasic manner: the active rRNA genes replicate early, whereas silent rDNA arrays replicate late [115, 127]. The association of NoRC with rRNA genes was shown to take place immediately after rDNA replication in late S-phase [115], suggesting a role of NoRC in maintaining the epigenetic and chromatin state of newly duplicated silent rRNA genes.

As described in the previous paragraph (**1.5.3**), an important event required for NoRC-mediated rDNA silent chromatin formation is the association of TIP5 with pRNA [90]. Recently, it was shown that synthesis of the pRNA-precursor IGS rRNA occurs during a restricted time window of S phase (early) and originates from the spacer promoter of a specific set of active and hypomethylated rRNA genes (**Figure 6**). Shortly after synthesis, IGS transcripts are processed (mid to late S-phase) to yield pRNA that is indispensable for establishment of silent rDNA chromatin mediated by NoRC [91]. This mode of action suggests that pRNA acts in *trans* to establish and propagate heterochromatin states of late-replicating silent rDNA copies. Notably, timing of IGS rRNA transcription (early S-phase) and processing (mid-late S-phase) into pRNA correlates well with the time when NoRC associates with newly replicated silent rRNA genes (mid-late S-phase) to re-establish silent chromatin (**Figure 6**) [115]. These findings

indicate that the cell carefully tunes the timing of IGS rRNA transcription/processing to inherit rDNA silencing during cell division and suggest that replication timing serves to coordinate synthesis and availability of factors (in this case pRNA) at the time when they have to bind selectively to newly replicated chromatin to propagate their epigenetic state to next cell generation (**Figure 6)** [97].

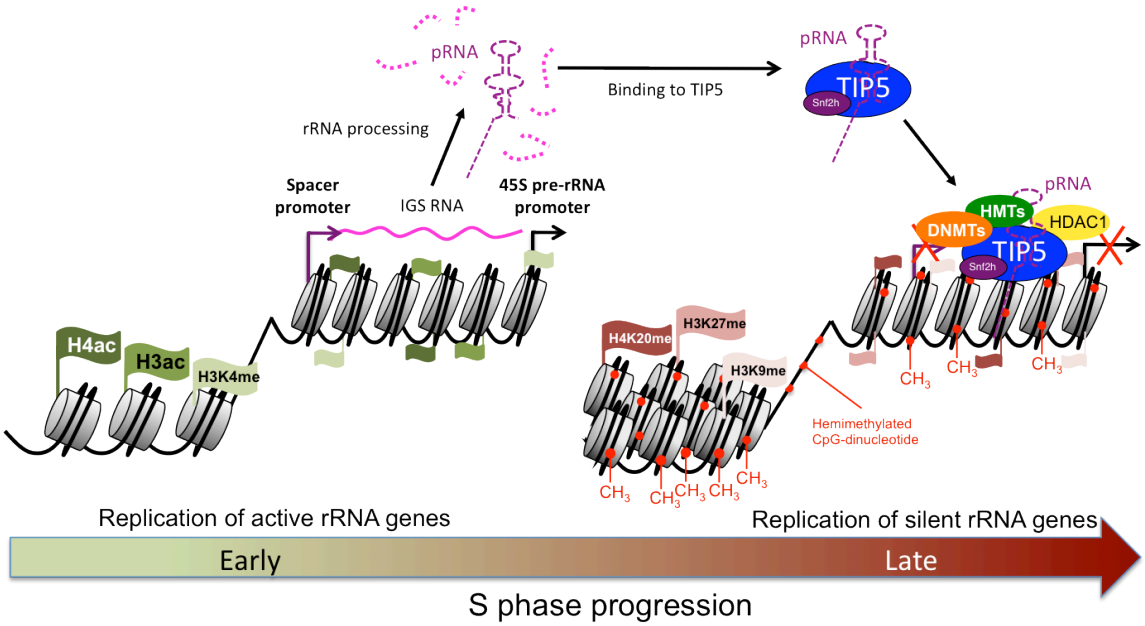

Figure 6 | Model showing inheritance of active and silent chromatin structure during S phase During early S-phase a subset of active genes transcribe IGS rRNA originating from the spacer promoter. Immediately after synthesis, IGS rRNA is processed during mid to late S-phase to yield pRNA that is indispensable for NoRC-dependent rDNA silencing. NoRC binds to pRNA, associates with newly late replicating genes and re-establish the silent chromatin structure by recruiting DNA and histone modifying enzymes.

Recent results revealed another layer of epigenetic control that involves acetylation state of TIP5 to modulate pRNA-NoRC association during S phase progression [121]. Acetylation at K633 of TIP5 was shown to fluctuate during S phase progression. At early S-phase, the acetyltransferase MOF acetylates TIP5 while at mid-late S-phase the acetyl moiety is displaced by the NAD^+-dependent deacetylase SIRT1 (Sirtuin 1). Acetylation of TIP5 was shown to decrease pRNA binding to TIP5 and to be required for positioning of the nucleosome over the rDNA promoter at the location characteristic of silent rRNA genes (-132/+22). The authors proposed a model where MOF-mediates acetylation of TIP5 during early S-phase and promotes nucleosome

positioning at the rDNA promoter which in turn allows methylation at CpG -133 at newly synthesized rDNA. However, we think that this model does not take into account that a positioned nucleosome cannot be maintained after the passage of the replication fork at the silent rDNA copies. Thus, although deacetylation of K633-TIP5 in mid-late S-phase correlates well with the timing of NoRC-pRNA binding to newly replicated silent rRNA genes, the proposed model is weak in offering explanations of the role of acetylated TIP5 in the time window of early-mid S phase that precedes replication of silent rRNA genes. Replication of DNA requires disruption of parental nucleosomes, implying that mechanisms must exist able to loose chromatin compaction and facilitate the disassembly of nucleosomes before passage of replication machinery. Following this line, acetylation of TIP5 and consequent weakening of NoRC-pRNA association and binding to rDNA can be part of this temporally coordinated changes aimed to decompact rDNA silent chromatin structure before passage of the replication fork. Similar chromatin structural changes have been also attributed to phosphorylation of histone H1 in late G1 and S phase that, by decreasing binding to nucleosomal DNA, might lead to a less compacted higher order chromatin structure [128-130]. As a consequence of this, it was proposed that the accessibility of pre-replication complex to the origin of replication and, probably, the initiation process itself through the chromatin barrier would be facilitated to some extent.

2 Aim of the thesis

Aim 1: Functional role of rDNA silencing and of NoRC complex in cell metabolism and nucleolus and nuclear architecture

As discussed in **1.5**, **1.6**, synthesis of rRNA transcripts represents a key event for ribosome biogenesis process. The presence of heterochromatic silent rDNA repeats raises the question about their function, which can be either to dose the rRNA transcript levels and/or to mediate the structure of the nucleolus and of other types of nearby localized heterochromatin like centric and pericentric repeats. Thus, understanding the role of rDNA silencing can have important consequences in cancer biology, offering unexplored opportunities for therapeutic intervention.

The specific aims of this work were to determine whether the levels of rDNA silencing and the NoRC complex affect:

1- rRNA transcript levels;
2- Cell growth and proliferation;
3- Nucleolus structure;
4- The organization of nuclear heterochromatin.

Aim 2: Analysis of the molecular mechanisms that mediate establishment and inheritance of silent rDNA chromatin

Recent studies revealed that the association of TIP5 with the non-coding pRNA plays a key role in the establishment of silent rDNA chromatin. However, the underlying mechanism of this process remained still elusive.

Hence, the second aim of this thesis was to determine how TIP5-pRNA association acts for the establishment and propagation of rDNA heterochromatin during cell division. Here we hypothesized that pRNA, once bound to TIP5, might serve as platform for the recruitment of chromatin repressor complexes.

The specific aims of this work were:

1- to identify protein factors that associated with TIP5 *via* pRNA;
2- to determine whether these protein bind to silent rRNA genes;
3- to determine whether the association with TIP5-pRNA is cell cycle specific;
4- to analyze the role of these identified factors in rRNA transcription and formation of silent rDNA chromatin.

3 Results

3.1 Overview of published and submitted manuscripts

3.1.1 The NoRC complex mediates the heterochromatin formation and stability of silent rRNA genes and centromeric repeats

Authors: Claudio Guetg, Philipp Lienemann, Valentina Sirri, Ingrid Grummt, Daniele Hernandez-Verdun, Michael O Hottiger, Martin Fussenegger and Raffaella Santoro

Journal: The EMBO Journal (2010) 29, 2135–2146

Link: http://www.nature.com/emboj/journal/v29/n13/full/emboj201017a.html

Contribution: Designing experiments, performance and analysis of the following figures: Fig. 2B-E, Fig. 3C, Fig. 4A-D, Fig. 5A, Suppl. Fig. 2A, Suppl. Fig. 3, Suppl. Fig. 4A-E; R.S. supervised the project and wrote together with C.G. the manuscript.

3.1.2 PARP1 is recruited to the rRNA genes *via* non-coding RNA and mediates inheritance of silent rDNA chromatin

Authors: Claudio Guetg, Fabian Scheifele, Florian Rosenthal, Michael O. Hottiger and Raffaella Santoro

Journal: submitted for publication

Link: not yet available (under revision)

Contribution: Designing experiments, performance and analysis of the following figures: Fig. 1A-C, Fig. 1E, Fig. 2B, Fig. 2D-H, Fig. 3A-G, Fig. 4A-D, Fig. 5A-E, Suppl. Fig. 2, Suppl. Fig. 3A-C, Suppl. Fig. 4A-D, Suppl. Fig. 5A-C, Suppl. Fig. 6, Suppl. Fig. 7A-B; Suppl. Fig. 8A-B;R.S. supervised the project and wrote together with C.G. the manuscript.

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THE EMBO JOURNAL

The NoRC complex mediates the heterochromatin formation and stability of silent rRNA genes and centromeric repeats

This article has been corrected since Advance Online Publication and a corrigendum is also printed in this issue.

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Maintenance of specific heterochromatic domains is crucial for genome stability. In eukaryotic cells, a fraction of the tandem-repeated ribosomal RNA (rRNA) genes is organized in the heterochromatic structures. The principal determinant of rDNA silencing is the nucleolar remodelling complex, NoRC, that consists of TIP5 (TTF-1-interacting protein-5) and the ATPase SNF2h. Here we showed that TIP5 not only mediates the establishment of rDNA silencing but also the formation of perinucleolar heterochromatin that contains centric and pericentric repeats. Our data indicated that the TIP5-mediated heterochromatin is indispensable for stability of silent rRNA genes and of major and minor satellite repeats. Moreover, depletion of TIP5 impairs rDNA silencing, upregulates rDNA transcription levels and induces cell transformation. These findings point to a role of TIP5 in protecting genome stability and suggest that it can play a role in the cellular transformation process.

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Subject Categories: chromatin & transcription; genome stability & dynamics
Keywords: cell proliferation; genomic stability; heterochromatin; NoRC; rDNA silencing

Introduction

Formation of specific heterochromatic domains is crucial for genome stability (Grewal and Jia, 2007; Peng and Karpen, 2008). This is exemplified by the heterochromatin structure of repetitive major satellite (pericentric) and minor satellite (centric) DNA sequences whose maintenance and accurate reproduction throughout multiple cell divisions represents a major challenge to ensure genome stability. In interphase, the centromeric heterochromatin is predominantly located either at the nuclear periphery or around the nucleolus (Haaf and Schmid, 1991; Pluta *et al*, 1995). The nucleolus is the subnuclear body where the tandemly repeated ribosomal RNA (rRNA) genes synthesize ribosomal RNA, the major components of the ribosome. In humans and apes, rRNA genes are located between the short arm and the satellite body of acrocentric chromosomes. Standard laboratory strains of mice, which are thought to have originated mainly from a European subspecies, *Mus musculus domesticus*, and partially from an Asian subspecies, *M.m.musculus/molossinus*, have rDNA clusters within the centromeric regions of chromosome-12, 15, 16, 18 and 19 (Dev *et al*, 1977; Davisson, 1989; Kurihara *et al*, 1994). However, the combinations of chromosomes that include rDNA have been shown to be highly polymorphic among individuals (Suzuki *et al*, 1990). Due to the linear proximity, centromeres of chromosomes bearing rDNA repeats associate with nucleoli. Notably, also chromosomes devoid of rRNA genes have their centromeres associated with the nucleolus at a frequency more than that expected for a random distribution (Carvalho *et al*, 2001 and references therein). The basis of this association probably relies on the linear proximity along the chromosome and on the repeated nature of DNA sequence, which provides multiple binding sites for specific proteins capable of forming multimeric complexes. In each cell, a fraction of rRNA genes is transcriptionally silent and organized in heterochromatic structures by epigenetic mechanisms (reviewed by Santoro, 2005). By contrast, the 'active' euchromatic fraction represents rRNA genes competent for transcription whose activity is modulated according to the requirement of cell metabolism (Grummt, 2003; Moss *et al*, 2007). CpG-methylated silent rRNA genes were shown to assemble adjacent to the perinucleolar heterochromatin in mouse neuronal cells, suggesting an intricate relationship between these heterochromatic regions and silent rRNA copies (Akhmanova *et al*, 2000).

The presence of heterochromatic silent rDNA repeats raises question regarding their function, which could be to either dose the rRNA transcript levels and/or to affect the structure of other types of chromatin localized nearby. Here we show that the role of TIP5 (TTF-1-interacting protein-5), the key subunit of the NoRC (nucleolar remodelling complex), is not only restricted to the formation of heterochromatin at the rDNA repeats, but that it also extends its action in establishing the perinucleolar heterochromatin and repressive histone marks at major and minor satellite sequences.

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The results indicate that TIP5 is crucial to maintain the stability of silent rRNA genes and centric and pericentric repeats. We also show that cells depleted of TIP5 upregulate rDNA transcription levels, increase cellular proliferation rates and have a transformed phenotype. The results uncovered a role of TIP5 in protecting genome stability and suggest that deregulation of rDNA silencing can contribute to cellular transformation.

Results

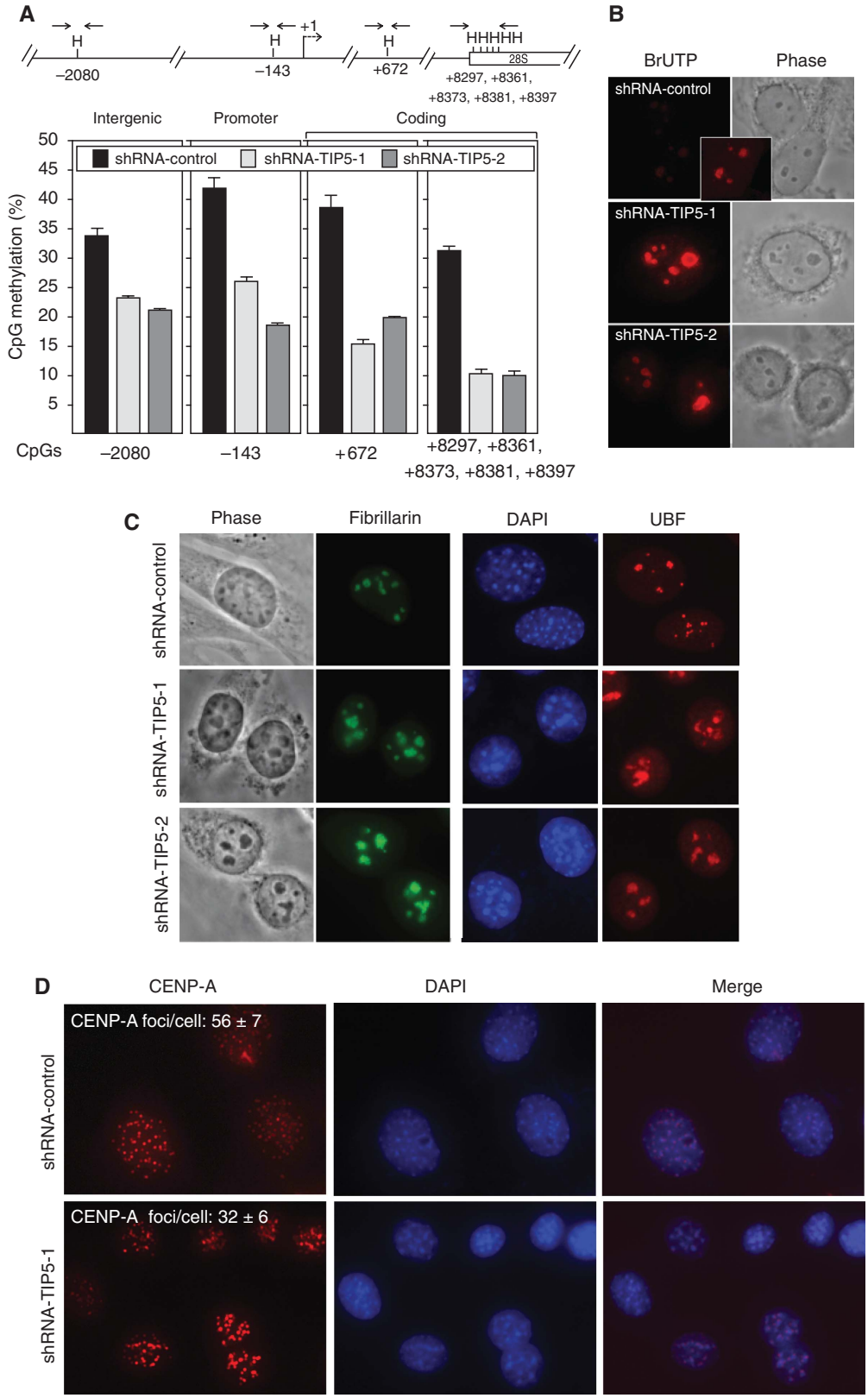
The principal determinant that establishes rDNA silencing is NoRC that consists of TIP5 and the ATPase SNF2h (Strohner *et al*, 2001; Santoro *et al*, 2002). NoRC binds to the rDNA promoter and represses rDNA transcription through recruitment of histone-modifying and DNA-methylating enzymes (Santoro *et al*, 2002; Zhou *et al*, 2002; Santoro and Grummt, 2005; Mayer *et al*, 2006). We have recently shown that depletion of TIP5, the NoRC subunit recruiting repressor complexes at the rDNA locus, affects rDNA silencing in mammalian cells (Santoro *et al*, 2009). Two cell lines (shRNA-TIP5-1 and shRNA-TIP5-2), derived from NIH3T3 cells, were established, each expressing a different shRNA sequence directed against TIP5 and both showing a reduction in TIP5 of about 80% when compared with a control cell line (Supplementary Figure S1A and B). Previous results showed that TIP5 binds to the rDNA promoter region and induces *de novo* methylation of these sequences (Santoro *et al*, 2002; Li *et al*, 2005). In NIH3T3 cells, about 40–50% of the rDNA promoter sequences are CpG-methylated. As shown in Figure 1A, rDNA CpG methylation levels were reduced over the entire rRNA gene in both shRNA-TIP5 cell lines when compared with that in control cells, underscoring the role of TIP5 in initiating local silencing events, which then spread over the whole rDNA unit (Figure 1A). Measurements of rRNA transcription by qRT–PCR and *in vivo* BrUTP incorporation showed higher levels of rRNA synthesis in both shRNA-TIP5 cell lines with respect to that in the control cell line (Supplementary Figure S1C and Figure 1B). Similarly, 45S pre-rRNA synthesis was enhanced in NIH3T3 cells 10 days after infection with a retrovirus expressing miRNA directed against TIP5 sequences (Supplementary Figure S1D). All these results indicate that depletion of TIP5 induces loss of rDNA silencing and enhances rRNA production.

TIP5 mediates the formation of perinucleolar heterochromatin

The nucleolus is the subnuclear body where rRNA is transcribed, processed and assembled into ribosomal subunits, and is a principal component of the nuclear architecture. Alterations in the nucleolar structure are often detected by changes in the levels of rRNA synthesis (Sirri *et al*, 2008). For example, as consequence of elevated nucleolar activities, cancer cells show enlarged nucleoli, which are commonly used by pathologists to identify tumour formation (White, 2005). To determine whether the nucleolar structure is affected in TIP5-depleted cells, we analysed the cellular localization of the nucleolar proteins fibrillarin or UBF (upstream binding factor) by immunofluorescence (Figure 1C). Statistical analyses of 100 shRNA-TIP5 and control cells selected at random showed that the nucleoli of shRNA-TIP5 cells diminished in number (shRNA-control cells: 6.09 ± 1.47 nucleoli per cell; shRNA-TIP5 cells: 3.90 ± 1.47 nucleoli per cell) and had enlarged structures (shRNA-control cells: $12.64 \pm 3.37\,\mu m^2$/cell; shRNA-TIP5 cells: $21.19 \pm 6.55\,\mu m^2$/cell), a characteristic indication of elevated rDNA transcription activities. Notably, TIP5-depleted cells showed an altered staining with DAPI (4,6-diamidino-2-phenylindole), a fluorochrome able to detect condensed heterochromatic loci (CC) formed by coalescence of centromeres in mouse interphase cells (reviewed by Maison and Almouzni, 2004). Whereas in control cells these CC are equally distributed all over the nucleus, in shRNA-TIP5 nuclei they are diminished in number and increased in size, indicating that structural changes occurred at these loci. Consistent with this, we detected structural alterations at the centromeric loci in interphase shRNA-TIP5 cells after immunostaining with antibodies against the core kinetochore CENP-A (Figure 1D) (reviewed by Black and Bassett, 2008). Although cellular amounts of CENP-A remain the same in both shRNA-control and shRNA-TIP5 cells (Supplementary Figure S2A), similar to the CC visualized by DAPI staining, the CENP-A-stained foci in TIP5-depleted cells were diminished in number (shRNA-control cells: 56 ± 7 per cell; shRNA-TIP5 cells: 32 ± 6 per cell) and increased in size. The heterochromatin of pericentric and centric regions were shown to localize also alongside the nucleolus (Carvalho *et al*, 2001; Guenatri *et al*, 2004 and Supplementary Figure S2B) and, in the specific case of mouse neuronal cells, even adjacent to methylated silent rRNA genes (Akhmanova *et al*, 2000). To analyse whether perinucleolar distribution of heterochromatin is affected in TIP5-depleted cells, we performed electron microscopic analysis (Figure 2A). The nucleolus is organized in three main structures: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC). Repressed rDNA genes are localized in the FC and initiation of rDNA transcription occurs at the FC–DFC boundary. The resulting pre-rRNA transcripts emerge into the DFC where they are cleaved and modified by the small nucleolar RNPs (snoRNPs) and processing enzymes (reviewed by Boisvert *et al*, 2007). Using a contrast specific for nucleic acids (Junéra *et al*, 1995), two kinds of heterochromatin (CC) with distinct positioning relative to the nucleolus were defined: intra-CC (ICC) in contact with the FC and extra-CC (ECC) at the nucleolar periphery (Figure 2A). In 13 shRNA-control and 14 shRNA-

Figure 1 Depletion of TIP5 impairs perinucleolar heterochromatin formation. (**A**) Depletion of TIP5 decreases CpG methylation at the entire rDNA repeat. A schema representing a single mouse rDNA repeat and the analysed *Hpa*II (H) sites. The arrows represent the primers used to amplify the *Hpa*II-digested DNA. The data represent the amounts of *Hpa*II-resistant rDNA normalized to the total rDNA calculated by amplification with primers encompassing DNA sequences lacking *Hpa*II sites and undigested DNA. The error bars indicate the s.d. of three independent experiments. (**B**) Depletion of TIP5 enhances rDNA transcription. rRNA transcripts were detected by *in situ* BrUTP incorporation after same exposure time. The inset shows a longer exposure of one control cell. (**C**) Depletion of TIP5 alters the number and size of nucleoli. Indirect immunofluorescence analysis of the nucleolar protein fibrillarin (left panel) or UBF (right panel) in shRNA-TIP5 and control cells. (**D**) Indirect immunofluorescence analysis of shRNA-control and shRNA-TIP5-1 cells using anti-CENP-A antibodies. The values represent the average number of CENP-A-stained foci of 50 cells scored at random.

TIP5 cells selected at random, the number of ICC and ECC was analysed. As rDNA transcription is initiated at the periphery of the FC, most probably the ICC in contact with the FC corresponds to the rDNA-bearing chromosomes and the ECC to chromosomes not containing rDNA repeats. The number of ICC was 84% in control cells and 4% in shRNA-TIP5 cells, whereas the ECC was observed in 70 and 56% of control and shRNA-TIP5 cells, respectively. All these results



indicated that depletion of TIP5 and reduction of rDNA silencing levels affects the nucleolar structure and the formation of condensed chromatin within and in close proximity of the nucleolus.

TIP5 mediates heterochromatin at centric and pericentric repeats

The centric and pericentric domains consist of repetitive minor and major satellite DNA repeats, respectively. These sequences are enriched in nucleosomes containing histones H3 tri-methylated at Lys9 (H3K9me3; Peters *et al*, 2003). To analyse whether TIP5 affects the epigenetic features at major and minor satellite repeats, we measured histone modifications by quantitative ChIP analysis (Figure 2B). Consistent with the CpG methylation results (Figure 1A), depletion of TIP5 increased the amounts of acetylated histone H4 (AcH4, active histone mark) at the rDNA promoter and decreased the levels of K20 trimethylation of histone H4 (H4K20me3, repressive histone mark), underscoring the role of TIP5 in establishing rDNA silencing. In contrast, the levels of H3K9me3 were slightly reduced. This is consistent with previous results showing that H3K9me3 is also present at active rRNA genes (Yuan *et al*, 2007). Notably, the levels of H3K9me3 and H4K20me3 were drastically reduced at both major and minor satellite sequences, whereas levels of histone acetylation were only slightly changed. These results indicated that impairment of heterochromatin formation by TIP5 depletion is not only restricted to the rDNA locus but also occurs at the centric and pericentric sequences. To determine whether increase in the levels of TIP5 affects the formation of repressive chromatin at these repeats, we overexpressed TIP5 in NIH3T3 cells using a retroviral TIP5-expression vector. As shown in Figure 2C, when TIP5 was overexpressed, the levels of H3K9me3 and H4K20me3 increased at the rDNA, major and minor satellite repeats but not at the α-globin genes. Notably, minor satellite repeats showed the most drastic changes, including a 40% reduction in AcH4 levels. All these results indicated that TIP5 modulates the formation of repressive chromatin not only at the rDNA locus but also at the major and minor satellite repeats.

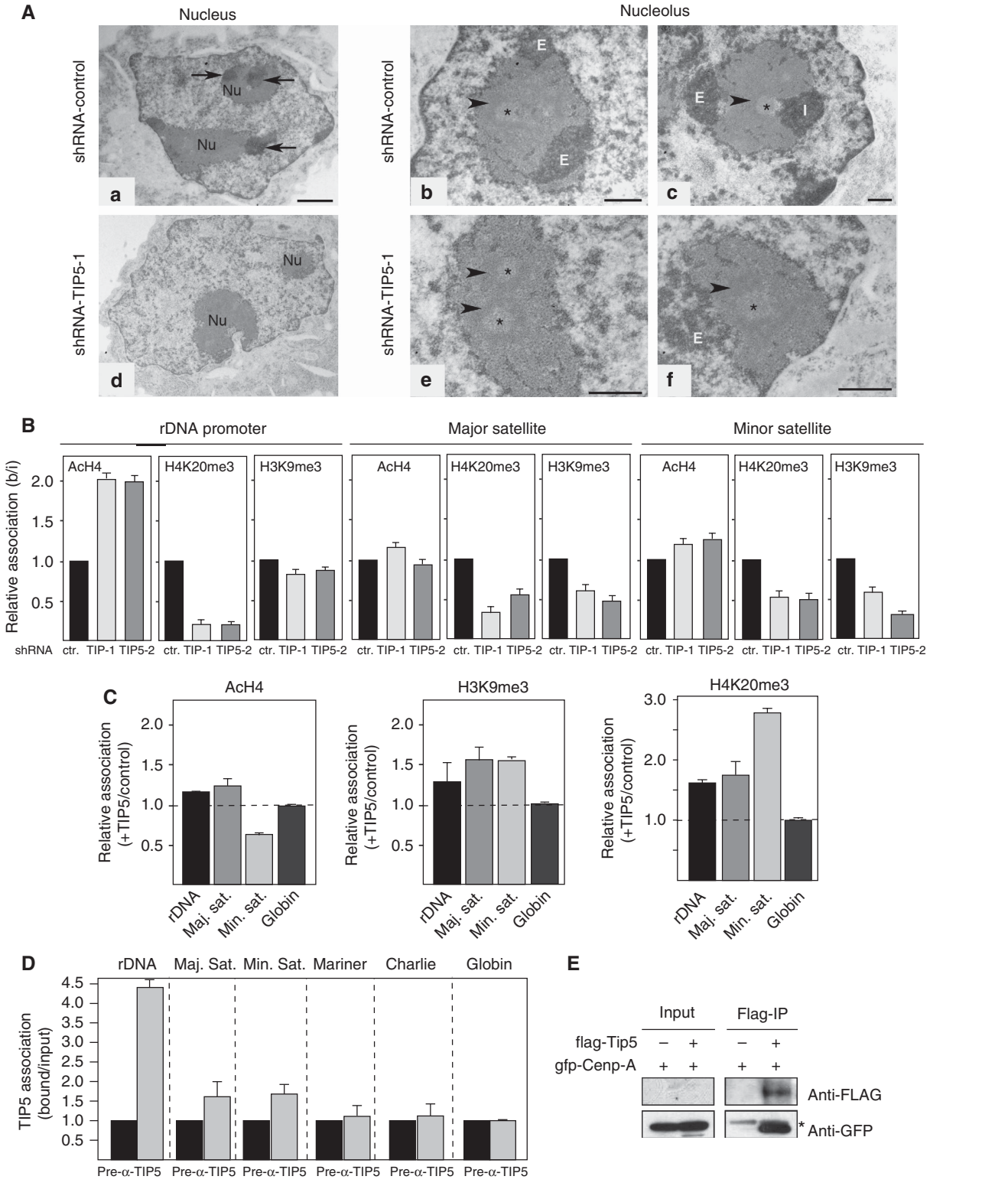
The results described so far suggest that TIP5 may bind to centric and pericentric repeats and establish heterochromatic structures using similar mechanisms used to silence the rDNA locus (Santoro *et al*, 2002; Zhou *et al*, 2002). Previous immunofluorescence studies showed that TIP5 localized exclusively within the nucleoli of NIH3T3 cells (Strohner *et al*, 2001). Colocalization of TIP5 with DAPI-stained heterochromatic loci, with the exception of those regions adjacent to the nucleolus, was never detected. To further assay whether TIP5 binds to centric and pericentric DNA, we performed ChIP assay. Consistent with previous results (Santoro *et al*, 2002), we found specific association of TIP5 with rRNA genes (Figure 2D). By contrast, the bound/input value of TIP5 immunoprecipitation (IP) with major and minor satellite repeats was much lower than that with rDNA sequences, although reproducibly higher when compared with a pre-immunoserum control IP and to a control α-globin gene and Mariner and Charlie transposon sequences. These results indicate that either TIP5 interacts with a minor fraction of centric–pericentric repeats or that this association is either weak and/or transient. To further examine the interaction of TIP5 with centric repeats, we analysed whether TIP5 associates with the core kinetochore protein CENP-A. After transfection of HEK293T cells with a plasmid expressing GFP-CENP-A with or without a FLAG-TIP5 expression vector, we detected anti-FLAG precipitated proteins on immunoblots using anti-GFP and anti-FLAG antibodies. As shown in Figure 2E, a significant amount of CENP-A was associated with TIP5. By contrast, no signal was detected when IP was performed in cells not expressing FLAG-TIP5. This result suggests that the interaction of TIP5 with centric repeats can be mediated by the association with the core kinetochore protein CENP-A.

TIP5 protects the stability of silent rDNA, centric and pericentric repeats

Repetitive DNA sequences, which constitute half of the genome in some organisms, often undergo homologous recombination, thus instigating genomic instability resulting from a gain or loss of DNA (Moazed, 2001; Grewal and Jia, 2007). Assembly of DNA repeats into a silent chromatin is generally thought to serve as mechanism ensuring repeat stability by limiting access to the recombination machinery. Consistent with this, in the budding yeast *Saccharomyces cerevisiae*, the stability of the rDNA repeats requires a Sir2-containing chromatin-silencing complex (Straight *et al*, 1999). Similarly, *Drosophila* Su(var)3-9 and RNAi mutants caused an increase in the amount of extrachromosomal circular rDNA, a typical result of rDNA recombination events (Peng and Karpen, 2007). The role of heterochromatic marks in preventing illicit DNA recombination events at repetitive sequences is also supported by data showing that the number

Figure 2 TIP5 mediates heterochromatin formation at major and minor satellites. (**A**) The distribution of the heterochromatin (CC) associated with the nucleoli of shRNA-control and shRNA-TIP5 cells. A general view of the nuclei (a, d) and the nucleoli (b, c, e, f). The contrast procedure reveals in dark the structures containing the nucleic acids, DNA or RNAs. (a) In the nucleus of an shRNA-control cell, the CC (arrows) are visible within the nucleolus (Nu) or at the nucleolar periphery. (d) In the nucleus of an shRNA-TIP5 cell, no CC are visible in or close to the nucleoli (Nu). (b, c) In shRNA-control nucleoli the CC are detected close (I, intra-CC) to the FC (*) or at the nucleolar periphery (E, extra-CC). (e, f) In shRNA-TIP5 nucleoli few CC are present (E). The arrowheads indicate the DFC (dense FC). Bar: (a, d) = 1 μm; (b, c, e, f) = 0.5 μm. (**B**) Depletion of TIP5 decreases repressive histone modification levels at the rDNA, major and minor satellite repeats. Quantitative ChIP analysis of cross-linked chromatin was precipitated with the indicated antibodies. The data are presented as the amounts of bound normalized to input and shRNA-control cell levels. The error bars indicate the s.d. of three independent experiments. (**C**) Overexpression of TIP5 modifies the heterochromatin of rDNA, major and minor satellites. The data are presented as a modified histone fold-change of NIH3T3 cells transiently transfected with TIP5-expression plasmids versus that in cells transfected with control plasmids. The error bars indicate the s.d. of two independent experiments. (**D**) ChIP showing association of TIP5 with a minor fraction of satellite repeats in NIH3T3 cells. The data are presented as the amounts of bound normalized to input and pre-immunoserum levels. The error bars indicate the s.d. of four independent experiments. (**E**) CENP-A interacts with TIP5 *in vivo*. HEK293T cells were co-transfected with GFP-tagged CENP-A plasmids in the presence and absence of pcDNA-FLAG-TIP5 and precipitated with anti-FLAG antibodies. Co-precipitated CENP-A was visualized on immunoblots using antibodies against GFP and FLAG. The signal indicated by the asterisk represents IgG. 10% of the lysate used for IP is shown (input). The low levels of FLAG-TIP5 in the input were below the detection limit.

of mouse minor satellite repeats decreased in *Dnmt1*-deficient cells (Jaco *et al*, 2008). To determine whether TIP5 protects genome stability, we depleted TIP5 in NIH3T3 cells for 10 days by miRNA, measured the amount of rDNA repeats by quantitative PCR and compared it to the levels of the α-globin gene (Figure 3A). In miRNA-TIP5 cells, we detected an about 20% reduction in the number of chromosomal rDNA copies as compared with that in the control cells, suggesting a role of TIP5 in preventing recombination events at the rDNA locus. Major and, more pronouncedly, minor satellite amounts were significantly reduced, indicating that as well these repeats are destabilized in the absence of TIP5.



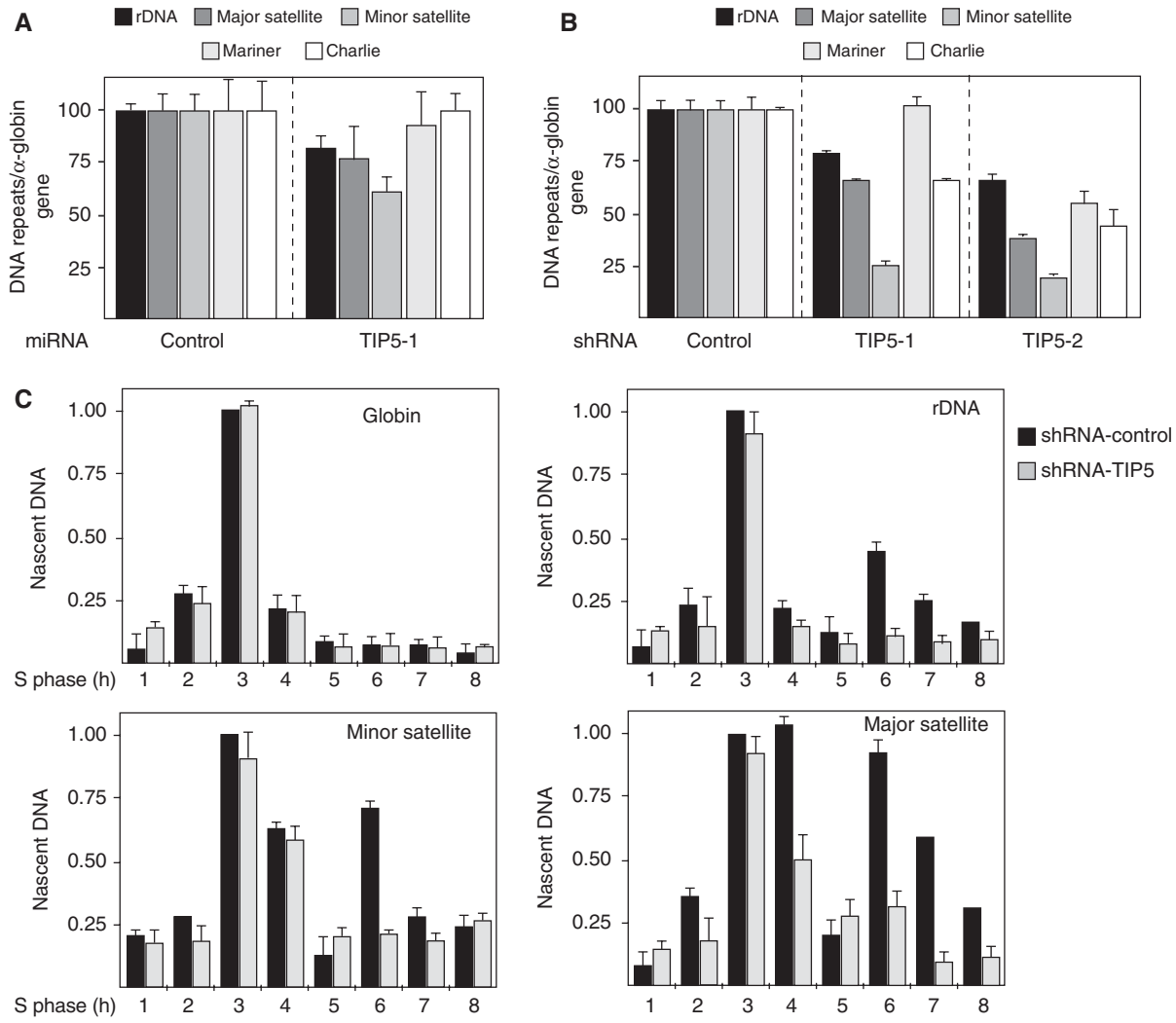


Figure 3 TIP5 protects genome stability. (**A**) Depletion of TIP5 induces loss of rDNA, major and minor satellite repeats. qPCR of genomic DNA from NIH3T3 cells infected for 10 days with a retrovirus expressing miRNA-TIP5 and (**B**) from shRNA-TIP5 cells. The values were normalized to the amounts of α-globin genes and to control cells. The error indicate the s.d. of three independent experiments. (**C**) Depletion of TIP5 alters the replication timing profiles of rDNA, major and minor satellite repeats. Synchronized cells were pulse-labelled with BrdU in 1-h intervals and nascent DNA was immunoprecipitated using anti-BrU antibodies. To calibrate for DNA recovery during IP, BrdU-labelled *E. coli* DNA was added to the reactions. Nascent DNA was measured by qPCR. The values represent the amounts of immunoprecipitated DNA normalized to the amounts of BrdU-labelled β-lactamase gene. The error bars indicate the s.d. of two independent experiments.

To determine whether this decrease in DNA levels was extended to other repeats, we analysed the amounts of the interspersed DNA transposons Mariner and Charlie. As shown in Figure 3A, both transposons were not substantially affected by TIP5 depletion, indicating that these are not the primary targets for TIP5. Consistent with these results, loss of rDNA, major and minor satellite repeats was also detected to a higher extent in shRNA-TIP5 stable cell lines (Figure 3B). However, in contrast to a short-time (10 days) TIP5 depletion, Charlie repeat levels diminished in both stable shRNA-TIP5 cells, whereas Mariner DNA levels decreased only in shRNA-TIP5-2, a likely effect of prolonged events of instability that the stable cell lines faced. All these results indicate that TIP5 protects the stability of rDNA, centric and pericentric repeats.

A typical feature of the heterochromatin is that its replication occurs usually in the late S-phase. The replication timing is tightly regulated and correlates with the chromatin states (Goren and Cedar, 2003). Consistent with this, rRNA genes show a biphasic replication profile: active genes replicate early whereas silent replicate late in S-phase (Li *et al*, 2005). To determine whether depletion of TIP5 induces loss of either early and/or late replicating repeats, we performed anti-BrdU IPs of nascent DNA from S-phase-synchronized cells pulse-labelled with 5-bromodeoxyuridine (BrdU). In both shRNA-control and shRNA-TIP5 cells, S-phase progression took about 7–8 h to complete and replication of the early replicating α-globin gene occurred 3 h after entry into S-phase (Figure 3C and Supplementary Figure S3). As expected, rDNA from control cells replicated both in early and mid-late S-phase (3 and 6 h) (Figure 3C). By contrast, the amounts of late replicating silent rDNA decreased in shRNA-TIP5 cells. Notably, the levels of early replicating rRNA genes did not increase proportionally, suggesting that depletion of TIP5 induces loss of rDNA repeats that replicate in late S-phase. Similarly, the levels of both late replicating major and minor

satellite repeats diminished in shRNA-TIP5 cells, whereas the levels of the early replicating fraction was not affected. All these results indicate that TIP5 protects the genome stability of repeats replicating in mid-late S-phase and suggest that TIP5 has a role in the duplication and inheritance of the chromatin state of rDNA, centric and pericentric repeats.

If TIP5 protects genome stability by establishing a heterochromatin at the rDNA repeats, depletion of TIP5 should instigate loss of silent CpG-methylated genes. To test this, we took advantage of the well-known presence of polymorphisms at the human and mouse rDNA sequences (Arnheim and Southern, 1977; Kominami *et al*, 1981). We tracked active and silent rRNA genes using a polymorphism that we found located at position +42/+43 (Figure 4A and Supplementary Figure S4A and B). We referred to these sequences as rDNA-A, rDNA-T and rDNA-G variants (v-rDNA). This polymorphism is conserved among mouse cells and tissues that we analysed so far (data not shown). To analyse the v-rDNA, we established polymorphism-specific rDNA amplifications that amplify specifically either rDNA-A or rDNA-G or rDNA-T sequences (Supplementary Figure S4C). NIH3T3 cells are characterized by a unique v-rDNA CpG methylation pattern that could not be found so far in other examined cell lines or tissues: about 85% of rDNA-A genes lack CpG methylation (i.e. active copies), whereas about 70 and 50% of rDNA-T and rDNA-G genes are CpG-methylated (i.e. silent copies) (Figure 4B and Supplementary Figure S4D). If the TIP5-mediated heterochromatin protects the stability of silent rRNA genes, the amounts of rDNA-A variants (85% unmethylated copies) should not be greatly affected by TIP5 depletion. To test this, we measured the amounts of v-rDNA in shRNA-TIP5 cells by quantitative PCR and compared it to the expression levels of the α-globin gene. As shown in Figure 4C, the levels of rDNA-G and rDNA-T genes decreased whereas rDNA-A copies were not affected, suggesting that stability of active variants is not influenced by TIP5. Notably, whereas the number of methylated silent genes decreases, the amounts of unmethylated rDNA-A, rDNA-T and total

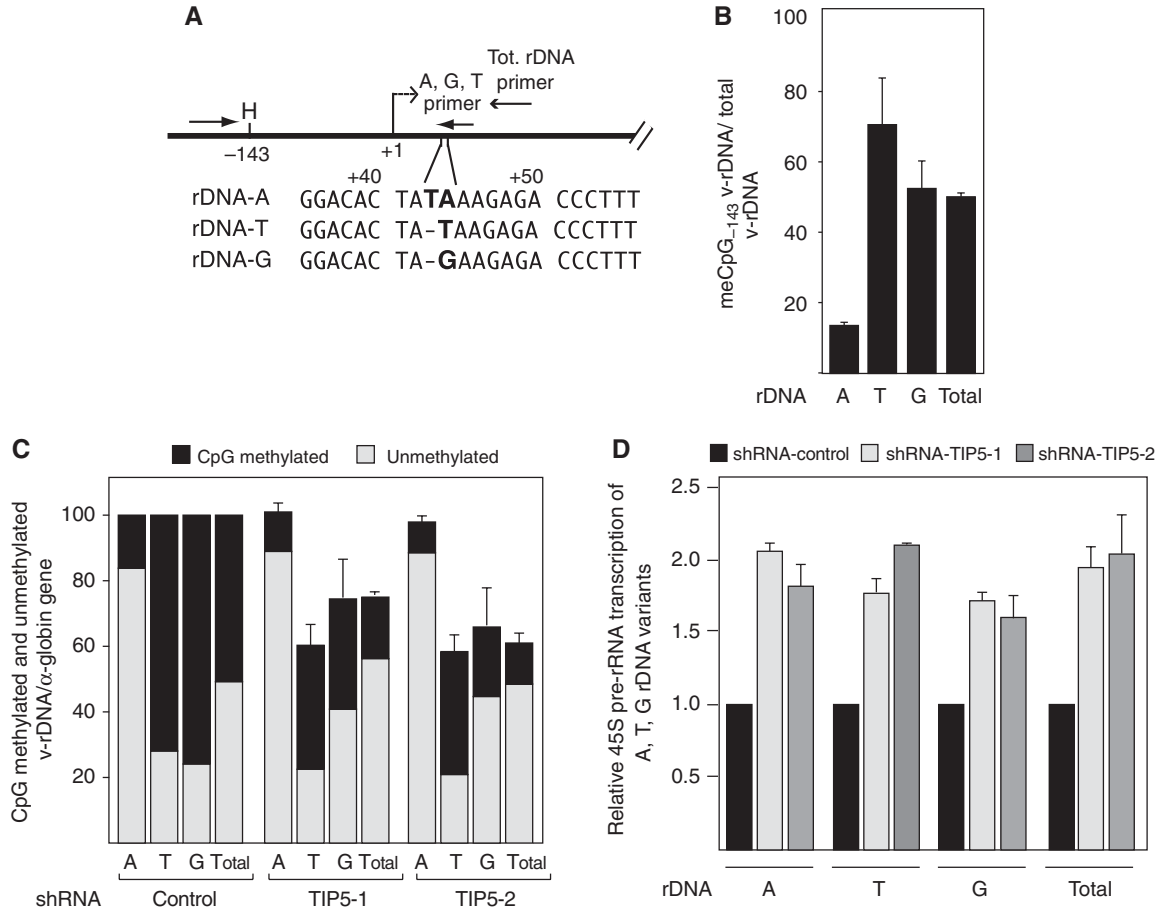


Figure 4 Depletion of TIP5 induces loss of CpG methylated, silent rDNA repeats. (**A**) A schema representing rDNA polymorphisms at +43/+44 (A, G and T sequences). The arrows represent the primers used to specifically amplify v-rDNA. (**B**) The CpG methylation profile of the v-rDNA promoter region in NIH3T3 cells. Polymorphism-specific qPCR. The data represent the amounts of *Hpa*II-resistant v-rDNA normalized to the corresponding total v-rDNA calculated by amplifications using primers encompassing v-DNA sequences lacking *Hpa*II sites and undigested DNA. The error bars indicate the s.d. of three independent experiments. (**C**) TIP5 mediates the stability of silent rRNA genes. Polymorphism-specific qPCR of v-rDNA from shRNA-TIP5 and control cells. The data were normalized to the amounts of α-globin genes and to control cells. Silent, methylated rDNA represents the *Hpa*II-resistent fraction relative to v-rDNA amounts. The *Hpa*II-digested fraction corresponds to active genes, lacking CpG methylation. The error bars indicate the s.d. of three independent experiments. (**D**) Depletion of TIP5 enhances the transcription of active rRNA genes. rRNA transcripts originating from v-rDNA variants were measured by qRT–PCR. The data are presented as the amounts of v-rRNA transcripts normalized to GAPDH mRNA levels and to control cells. The error bars indicate the s.d. of four independent experiments.

genes remain relatively unchanged, underscoring the role of TIP5-mediated rDNA heterochromatin in protecting the genome stability of silent rRNA genes.

Changes in the amounts of silent and active rRNA genes is generally discussed as a mechanism that cells use to modulate and dose rRNA transcript levels (reviewed by Santoro, 2005 and McStay, 2006). However, until now, there was no satisfying correlation between the levels of synthesized 45S pre-rRNA transcripts and the number of rRNA genes (French *et al*, 2003). In agreement with these results, the data described so far indicate that rDNA transcription is upregulated in TIP5-depleted cells (Figure 1B and Supplementary Figures S1C and D), although the number of unmethylated, active genes is the same as that in the control cells (Figure 4C). These results suggest that enhancement of rDNA transcription in TIP5-depleted cells does not depend on the number of active genes. To further investigate this point, we compared the levels of rRNA transcripts synthesized by each class of rDNA variants. As shown in Figure 4D and Supplementary Figure S4D, all the variants, including the rDNA-A genes whose copy number was not affected by depletion of TIP5, transcribed at higher levels. These results strengthen the view that rDNA transcription is preferentially modulated by altering the transcriptional activity of each gene and not by altering the number of genes. Moreover, the data imply that TIP5 and the levels of rDNA silencing influence and modulate the transcription rate of active rRNA genes.

Depletion of TIP5 induces cellular transformation

As genome instability and elevated rDNA transcription are typical features of cancer cells, we tested whether TIP5 can contribute to cellular transformation. As shown in Figure 5A, the population of both shRNA-TIP5 and miRNA-TIP5 cells decreased in the G_1/S and accumulated in the S and G_2-phases. Consistent with these results, shRNA TIP5 cells

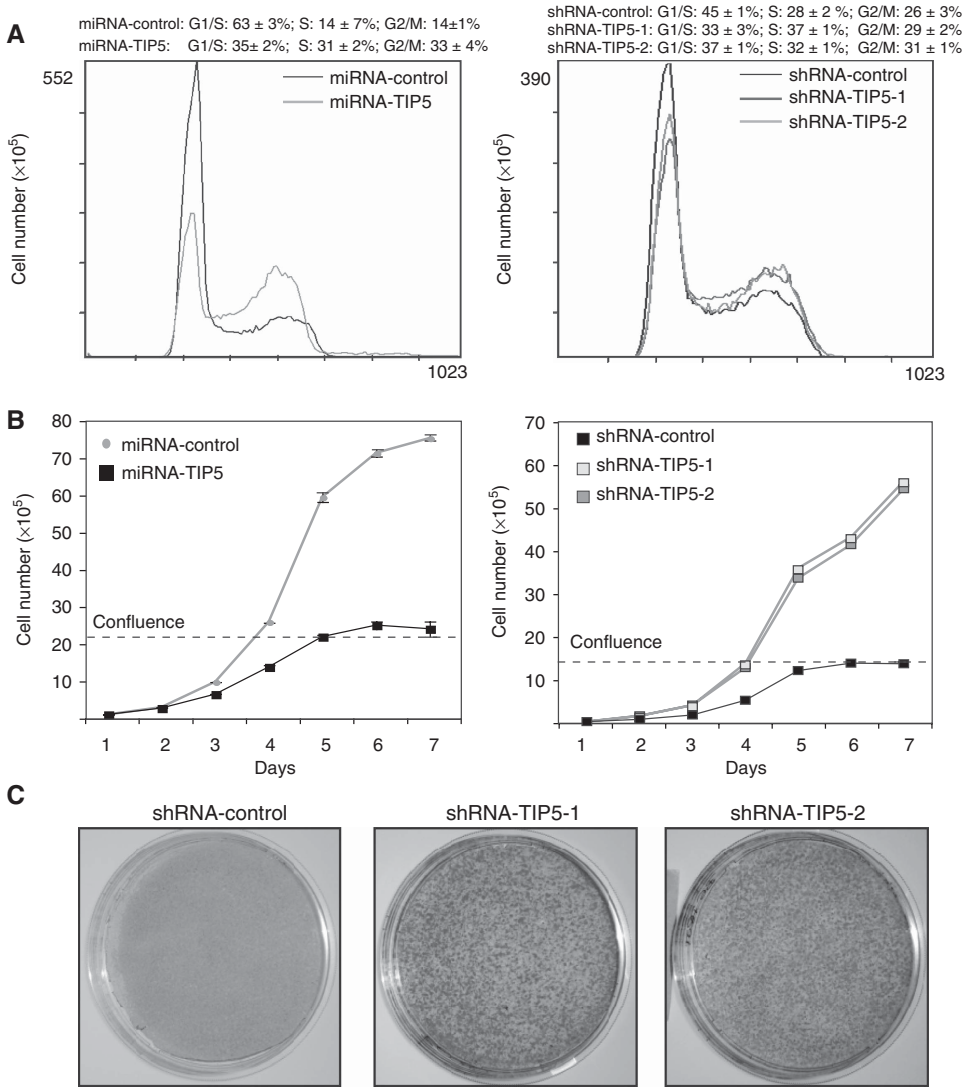


Figure 5 Depletion of TIP5 induces cellular transformation. (**A**) FACS analysis of miRNA and shRNA-TIP5 cells. Data were quantified from two independent experiments. (**B**) The growth curve of miRNA-TIP5 and shRNA-TIP5 and control cells. Cellular confluence was reached at about day 5. Similar results were obtained in three independent experiments performed in triplicates. The error bar values of shRNA cells are hidden by symbols. (**C**) The transforming activity of TIP5 depletion. Cells were plated at low density on a 10-cm-diameter plate. After 14 days (8 days after confluence) cells were stained with methylene blue.

showed increased incorporation of BrdU into nascent DNA and higher levels of cyclin-A (Supplementary Figures S5A and B). Comparison of the proliferation rates indicated that shRNA-TIP5 and miRNA-TIP5 cells proliferate faster than control cells, suggesting a functional link between TIP5 and control of cell growth and proliferation (Figure 5B). Notably, TIP5-depleted cells showed a transformed phenotype, continuing to proliferate beyond confluence, forming cellular foci and peeling off the culture surface in large mass (Figures 5B and C). A similar phenotype was obtained with NIH3T3 cells transformed with known oncoproteins like Ras (Tognon *et al*, 1998). All these results indicate that depletion of TIP5 can contribute to cellular transformation and strengthen the intimate link between rDNA transcription, genome instability and cancer.

Discussion

Our study provides insights into the role of TIP5 and rDNA silencing in mammalian cells. The results indicate that both depletion and overexpression of TIP5 affect heterochromatin formation at rDNA, centric and pericentric repeats, implying an intimate relationship that links TIP5 with rDNA silencing and formation of centromeric heterochromatin. In mouse and human chromosomes, the rRNA genes are positioned very close to the centromeres (Henderson *et al*, 1974; Elsevier and Ruddle, 1975) and centromeres of chromosomes bearing rDNA repeats associate with the nucleolus (Carvalho *et al*, 2001). Importantly, silent methylated rRNA genes were found localized in proximity to the centromeric heterochromatin in mouse neuronal cells (Akhmanova *et al*, 2000). According to our data, such spatial and linear closeness may allow TIP5, bound to silent rRNA genes, to interact with centric repeats and to aid in establishing heterochromatic structures using similar mechanisms as used to silence the rDNA locus (Santoro *et al*, 2002; Zhou *et al*, 2002) (Figures 6A and B). Although our ChIP data showed that this interaction is probably weak and transient, the association of TIP5 with the centromeric protein CENP-A suggested that this interaction indeed takes place. Alternatively, the repressive chromatin of silent rDNA copies may affect the centric and pericentric heterochromatin either by spreading mechanisms or by creating a nucleolar/perinucleolar compartment enriched in chromatin repressor complexes. In both cases, decrease of rDNA silencing after TIP5 depletion would affect the spreading of heterochromatin and reduce the levels of repressor complexes within and nearby the nucleolus. Notably, a role of the perinucleolar compartment in mediating the incorporation of repressive chromatin factors was recently discussed for the establishment of the inactive X-chromosome that contacts the nucleolus during mid-to-late S-phase to faithfully duplicate its epigenetic character (Zhang *et al*, 2007). Future studies will investigate whether formation of inactive X-chromatin at the nucleolar periphery is also a process that may depend on the levels of rDNA silencing and TIP5.

Our data show that TIP5 is involved in maintaining genome stability. In the yeast *S. cerevisiae*, recruitment of the nucleolar protein complexes RENT (regulator of nucleolar silencing and telophase exit) and Cohibin to rDNA suppresses unequal recombination at the rDNA repeats (Mekhail *et al*, 2008 and references herein). This suppression is seemingly

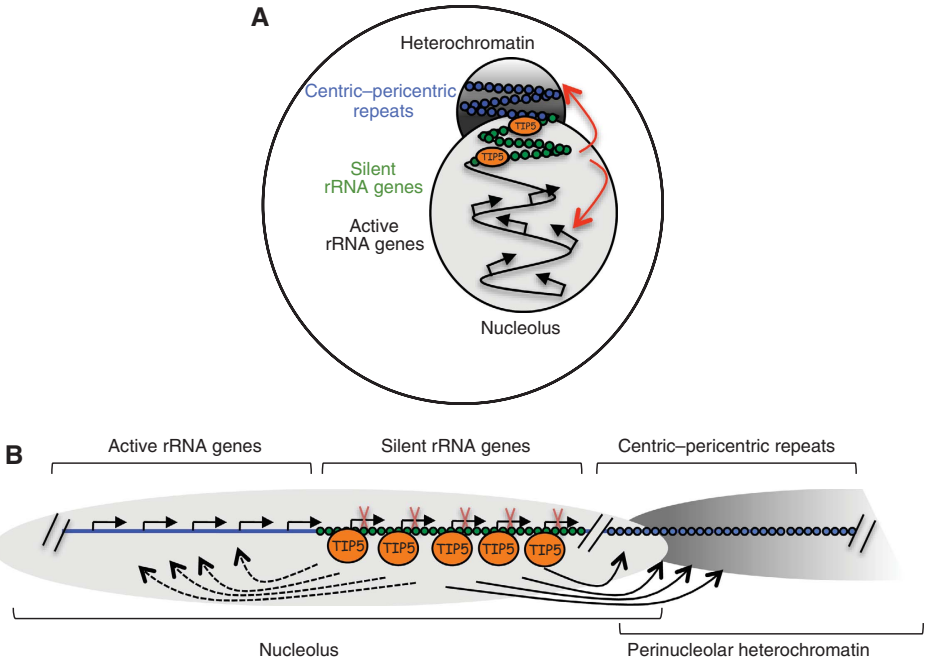


Figure 6 TIP5 mediates the heterochromatin at the nucleolar/perinucleolar associated chromatin. A model showing the role of TIP5 in establishing heterochromatin at regions located adjacent to the nucleolus. The cellular (**A**) and linear (**B**) distribution of active/silent rRNA genes and centromeric heterochromatin within the nucleolus and at the perinuclear periphery. In a transient association model, TIP5 interacts transiently and/or weakly with nearby localized chromatin domains (centric-pericentric repeats) from its stable binding sites (silent rRNA genes). Alternatively, spread of heterochromatin from silent rRNA genes or formation of nucleolar/perinucleolar compartment enriched in chromatin repressor complexes can affect the perinucleolar heterochromatin. In this model, it is also proposed that TIP5 and silent rRNA copies have a role in mediating the transcriptional activity of active rRNA genes as suggested by the results shown in Figure 4D.

linked to the ability of these complexes to induce rDNA silencing. Until now, the repetitive nature of the rRNA genes represented a limit in determining which rRNA genes (active or silent) undergo instability in the absence of these chromatin repressor complexes. By tracking rRNA genes with polymorphic variations, we showed that TIP5-mediated heterochromatin formation specifically protects silent rRNA genes from illicit recombination events whereas active genes are not affected. Similarly, our data showed that the stability of major and minor satellite sequences depends on TIP5. Loss of rDNA, major and minor satellite repeats is restricted to sequences replicating in the mid-late S-phase. This is consistent with previous results showing that TIP5 binds to rRNA genes, which replicate in the second half of S-phase (Li *et al*, 2005). Furthermore, it suggests that TIP5 is involved in the heterochromatin formation of centric repeats replicating in mid-late S-phase. As formation and maintenance of heterochromatic structures at these repeats are crucial for genome stability (Peters *et al*, 2001), our results show that the TIP5-mediated heterochromatin has an important role in protecting the genome from inappropriate chromosomal rearrangements.

Cells in the absence of TIP5 proliferated beyond confluence and had a transformed phenotype, a likely result of the genome instability that we detected in TIP5-depleted cells. Loss of genome stability is known to be a principal molecular step in cancer formation, contributing importantly to rapid selection of clonal cell populations that are able to overcome the various environmental challenges that arise during carcinogenic progression. In addition, in cancer cells, rDNA transcription is enhanced, contributing to increased production of ribosomes and protein synthesis of the rapidly proliferating tumours (Ruggero and Pandolfi, 2003; White, 2005). Disruption in one or more of the steps that control protein biosynthesis has been associated with alterations in the cell cycle and regulation of cell growth (White, 2005). Consistent with this, we have recently shown that depletion of TIP5 and impairment of rDNA silencing enhances ribosome synthesis and increases protein production (Santoro *et al*, 2009). A lower content of rDNA methylation was reported for several tumours (Qu *et al*, 1999; Shiraishi *et al*, 1999; Ghoshal *et al*, 2004), strengthening the notion of the role of CpG methylation in repressing rDNA transcription (Santoro and Grummt, 2001). Moreover, rDNA CpG methylation levels were found to be higher in ovarian cancer patients with long progression survival as compared with that in patients with short survival, an indication that rDNA silencing levels may influence cell growth properties essential for active tumour proliferation and tumour aggressiveness (Powell *et al*, 2002). Surprisingly, upregulation of rDNA transcription in TIP5-depleted cells does not depend on the de-repression of silent genes. Whereas the amount of silent genes decreases in these cells, the number of unmethylated active genes is not affected. Consistent with this, a specific class of rDNA variants (rDNA-A) synthesized higher rRNA transcript levels after TIP5 depletion, although the majority of these genes are active and their stability is not affected by TIP5. It seems, therefore, that TIP5 and/or presence of heterochromatic silent repeats indirectly affects the transcription rate of active genes, probably by enriching the nucleolar compartment of the chromatin repressor complexes. However, we cannot exclude the possibility that upregulation of rDNA transcription is a consequence of genome instability that caused the acquisition of aberrant mechanisms of rDNA transcriptional regulation, thus representing an advantage for the elevated protein synthesis necessary for high proliferative rates.

Although it remains to be estimated to which extent the genome instability or enhancement of rDNA transcription in TIP5-depleted cells contributed to the transformed phenotype, our results provide evidences that the TIP5-mediated heterochromatin has a crucial role in protecting genome stability and regulating rDNA transcription, thus contributing to the cellular transformation process.

Materials and methods

Stable and transient TIP5 knockdown

NIH3T3 cells were stably transfected with plasmids expressing shRNA-TIP5-1 (5′-GGACGATAAAGCAAAGATGTTCAAGAGACATCT TTGCTTTATCGTCC) and shRNA-TIP5-2 (5′-GCAGCCCAGGGAAAC TAGATTCAAGAGATCTAGTTTCCCTGGGCTGC) sequences under the control of the H1 promoter. Plasmids expressing control miRNA or miRNA targeting TIP5 (5′-GATCAGCCGCAAACTCCTCTGAGTTTTG GCCACTGACTGACTCAGAGGATTGCGGCTGAT) were constructed according to the Block-iT Pol II miR RNAi system (Invitrogen). Infections were performed according to manufacturer's instructions. Cells were analysed 10 days after infection.

Transcription and ChIP analysis

45S pre-rRNA transcription was measured by qRT–PCR in accordance with the standard procedure using the Universal Master mix (Diagenode). The primer sequences used to detect 45S pre-rRNA and GAPDH were as reported by Santoro and Grummt (2005). The rDNA transcription levels were normalized to GAPDH mRNA levels. ChIP analysis was performed as previously described (Santoro *et al*, 2002). rDNA, major and minor satellite sequences were amplified with previously reported primers (Santoro *et al*, 2002; Martens *et al*, 2005). rDNA methylation was measured as previously described (Santoro *et al*, 2002).

Indirect immunofluorescence

In vivo BrUTP incorporation was performed as previously described (Grob *et al*, 2009). To detect fibrillarin and UBF, cells were fixed in methanol for 20 min at −20°C, air-dried for 5 min and rehydrated with PBS for 5 min. Incubations with antibodies were performed as previously described (Strohner *et al*, 2001). DNA was stained with DAPI (Molecular Probes). The area of nucleoli was quantified using ImageJ software (NIH).

Electron microscopy

The DNAs and RNAs were contrasted with uranyl after methylation and acetylation of the amino and carboxyl groups as described by Junéra *et al* (1995). Briefly, cell pellets were fixed in glutaraldehyde, incubated in methanol and acetic anhydride, and embedded in Epon. The sections were contrasted by uranyl acetate for 60 min at RT. The specificity of the contrast was verified on ribosomes.

Immunoprecipitation

To monitor the interaction of TIP5 and CENP-A *in vivo*, we transfected HEK293T cells with expression vectors encoding the respective proteins (pcDNA-Flag-Tip5 and GFP-CENP-A (gift from K Sullivan)). After 48 h, we lysed the cells in a lysis buffer (50 mM Tris–HCl (pH 7.8), 150 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 20% glycerol, 0.1% NP-40, 0.1 mM PMSF, proteinase inhibitor cocktail (Roche)) at 4°C for 30 min. The cleared lysate was subjected to IP overnight at 4°C using an immobilized antibody against FLAG (anti-FLAG M2 affinity gel; Sigma). The precipitates were washed three times with a buffer containing 150 mM KCl, separated on either 6 or 12% SDS–polyacrylamide gels and analysed on western blots using anti-FLAG M2 (Sigma) and anti-GFP (Roche) antibodies.

Replication timing

Cells were synchronized at the G_1/S phase as previously reported (Li *et al*, 2005). The synchronized cells were pulse-labelled (30 min) with 30 μM 5′-BrdU in 1-h intervals. Nascent DNA was isolated,

purified and measured by qPCR. To calibrate DNA recovery, BrdU-labelled *Escherichia coli* DNA was added to the reactions before IPs. Semi-quantitative PCRs were normalized to the amounts of β-lactamase calculated by qPCR.

DNA copy number

Repetitive DNA sequences were quantitatively amplified from logarithmic dilutions of genomic DNA using previously reported primer sequences (Santoro *et al*, 2002; Martens *et al*, 2005). The data were normalized to the amounts of the α-globin gene.

Polymorphism-specific PCR

The following primers pairs were used to specifically amplify the v-rDNA variants: rDNA-A (+63/+42) Rev: TAAATCGAAAGGGT CTCTTT; rDNA-T (+62/+41) Rev: TAAATCGAAAGGGTCTCTTA; rDNA-G (+62/+41) Rev: TAAATCGAAAGGGTCTCTTC; total rDNA (+87/+66) Rev: TAGGCTGGACAAGCAAAACAG; total rDNA (+1/+20) For: ACTGACACGCTGTCCTTTCC; total rDNA (−165/−145) For: GACCAGTTGTTCCTTTGAGG.

Antibodies

Anti-TIP5 antibodies were purchased from Diagenode. Anti-acetylated histone H4, anti-H3K9me3 and H4K20me3 were obtained from Upstate. Anti-UBF and anti-cyclin-A antibodies were from Santa Cruz Biotechnology. A previously characterized human autoimmune serum with specificity against fibrillarin was used (Sirri *et al*, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Figures

Supplementary Figure S1

Depletion of TIP5 upregulates 45S pre-rRNA transcription levels. (**A**) Western blot of nuclei extracts from shRNA-TIP-1 and -2 and shRNA control cells using anti-TIP5 and anti-UBF antibodies. (**B**) qRT-PCR of TIP5 mRNA from shRNA-TIP5 and control cells. Values were normalized to GAPDH mRNA levels. Depletion of TIP5 enhances rDNA transcription. qRT-PCR. 45S pre-rRNA levels in stable shRNA-TIP5 cells (**C**) and in NIH3T3 10 days after infection with a retrovirus expressing miRNA sequences directed against TIP5 (**D**). Values were normalized to GAPDH mRNA levels and to control cells. Error bars are defined as the s.d. of three independent experiments.

Supplementary Figure S2

(**A**) Western blot showing similar levels of CENP-A protein in both shRNA-control and shRNA-TIP5 cells. To normalize protein loading, the levels of tubulin were monitored using an anti-tubulin antibody. (**B**) Cellular localization of centromers in NIH3T3 cells. Indirect immunofluorescence analysis of NIH3T3 cells with anti-UBF and anti-CENP-A antibodies. The merge panel shows co-localization of some centromers with the perinucleolar periphery.

Supplementary Figure S3

Profile of S-phase progression of shRNA-TIP5 and control cells. FACS analysis. Cells were maintained at confluence in DMEM/10% FCS for 2 days before reseeding ($2 \cdot 10^6$ cells in a 10 cm diameter dish) and culturing for 18 h in medium containing 1 μg/ml aphidicolin (Sigma) to arrest cells at G1/S phase boundary. After release from the aphidicolin block, cells were collected at the indicated times for FACS analysis.

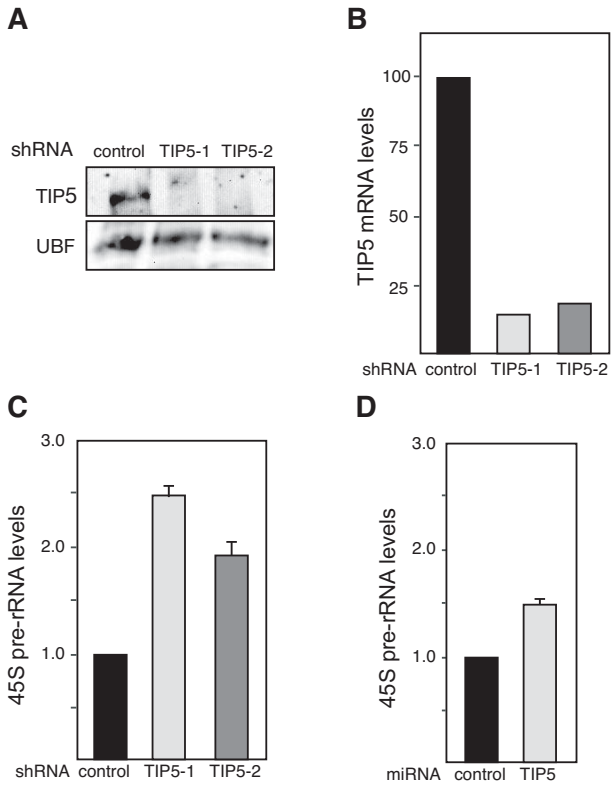
Supplementary Figure S4

rDNA polymorphism at +42/+43 marks rDNA variants. (**A**) Schema representing rDNA polymorfisms at +42/+43 (A, T and G sequences). Arrows indicate PCR primers used to amplify rDNA sequences from -165 to +83 (total rDNA) and from -165 to +64 (A, T and

G-sequences). (**B**) rDNA variant sequences. rDNA sequences were amplified from -1 to +155 from NIH3T3 genomic DNA. PCR product was directly sequenced and the region from +32 to +50 is shown. Sequences of plasmids containing rDNA-A and -T are shown. (**C**) Establishment of a polymorphic-specific qPCR. Specificity of the primers was assayed by amplification of v-rDNA plasmids. Values were normalized to the amounts amplified with total rDNA primer. (**D**) CpG methylation profile of v-rDNA variants from mouse liver (L), brain (B) and heart (H). (**E**) Depletion of TIP5 upregulates rDNA transcription at all v-rDNA genes. qRT-PCR from rRNA synthesized by v-rDNA in NIH3T3 cells 10 days after infection with a retrovirus expressing miRNA-TIP5. Data were normalized to GAPDH mRNA levels and to control cells.

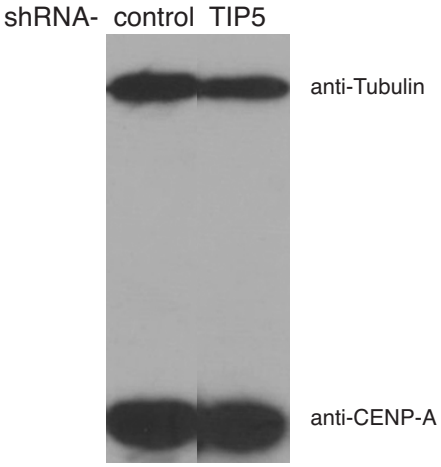
Supplementary Figure S5

(**A**) BrdU incorporation assay. Cells were incubated with 10 μM BrdU for 30 min, stained with antibodies against BrdU, and percentage of cells in S phase of two independent experiments was estimated. (**B**) Western blot of cellular lysates from shRNA-control and shRNA-TIP5 cells using antibodies against Cyclin A. Anti-actin antibodies were used to ensure that equal amounts of proteins were analyzed.



Supplementary Figure S1

A



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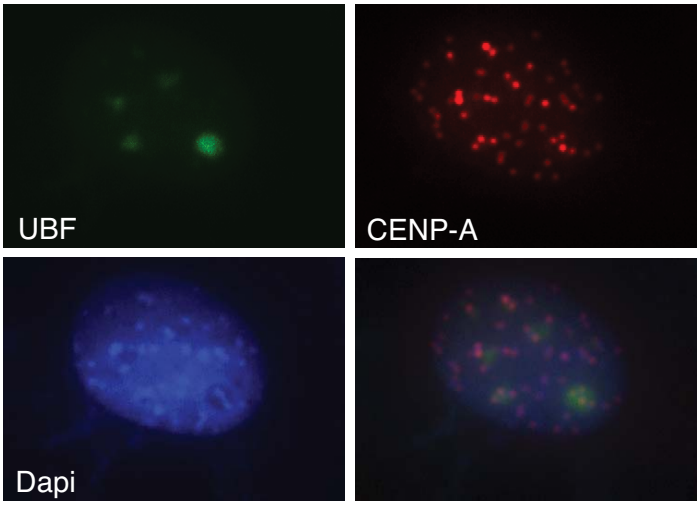
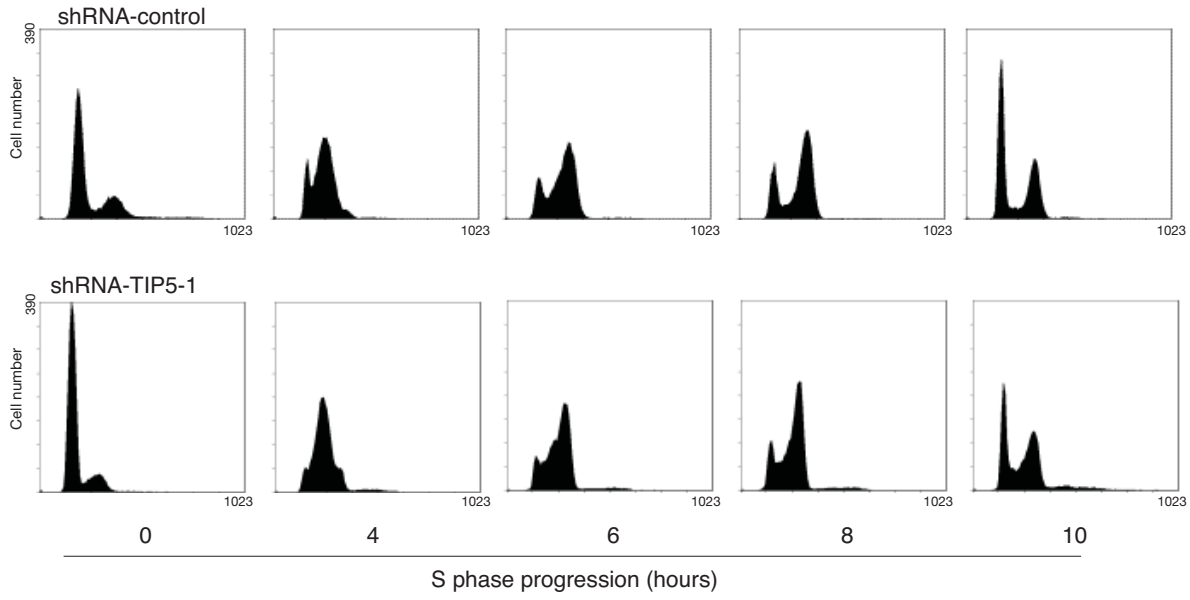
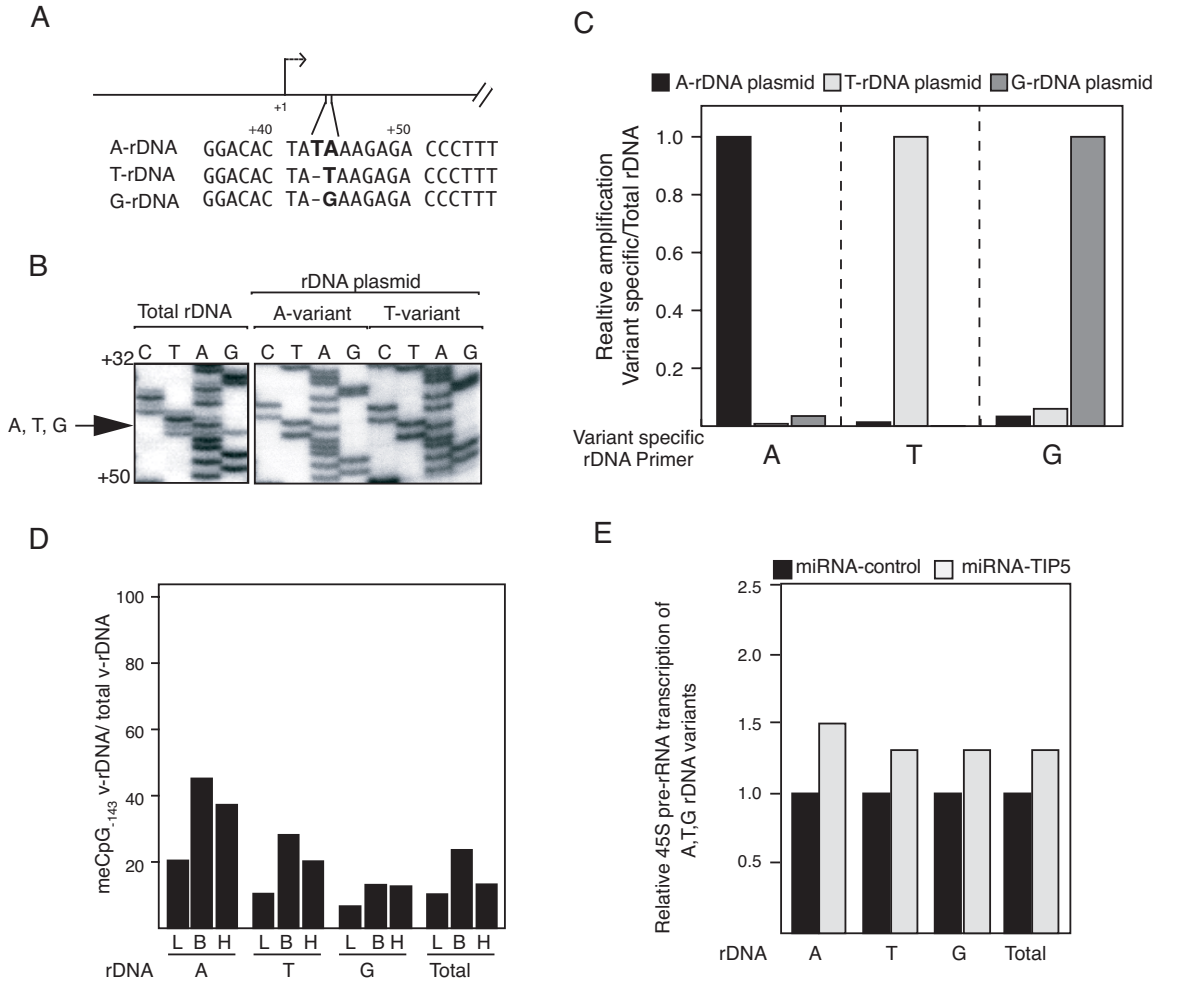


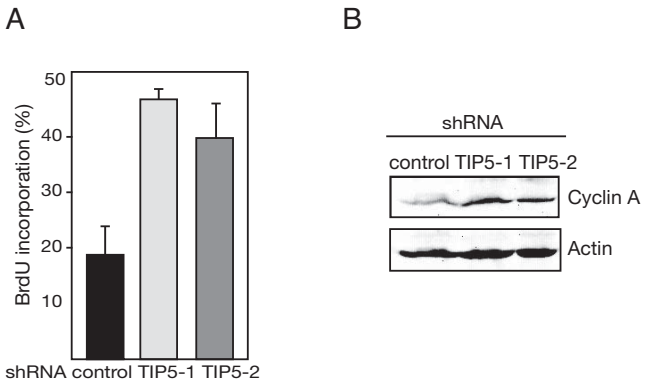
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5

3.1.2 PARP1 is recruited to the rRNA genes *via* non-coding RNA and mediates inheritance of silent rDNA chromatin

Inheritance of silent rDNA chromatin is mediated by PARP1 *via* non-coding RNA

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Abstract

Faithful propagation of specific chromatin states requires re-establishment of epigenetic marks after every cell division. How the original epigenetic signature is inherited after disruption during DNA replication is still poorly understood. Here we show that the poly(ADP-ribose)-polymerase-1 (PARP1/ARTD1) is implicated in the maintenance of silent rDNA chromatin during cell division. We demonstrate that PARP1 associates with TIP5, a subunit of the NoRC complex, *via* the non-coding pRNA and binds to silent rRNA genes after their replication in mid-late S-phase. PARP1 represses rRNA transcription and is implicated in the formation of silent rDNA chromatin. Silent rDNA chromatin is a specific substrate for ADP-ribosylation and the enzymatic activity of PARP1 is necessary to establish rDNA silencing. The data unravel a novel function of PARP1 and ADP-ribosylation that serves to inherit silent chromatin structures, shedding light on how epigenetic marks are transmitted during each cell cycle.

Maintenance and transmission of proper chromatin organization is fundamental for genome stability and function in eukaryotes. During DNA replication, both heterochromatin and euchromatin are disrupted ahead of the replication fork and are then reassembled into their original epigenetic states behind the fork. How chromatin domains are restored on new DNA and transmitted through mitotic cell division remains a fundamental question in biology, with implications for development and complex diseases like cancer (Jasencakova and Groth, 2011). In higher eukaryotes, the tandemly repeated ribosomal RNA (rRNA) genes represent a striking example of how specific chromatin states are propagated during cell cycle. In each cell, a fraction of the rRNA genes is transcriptionally silent, replicates in late S-phase and is organized in heterochromatic structures by epigenetic mechanisms, including silent histone marks and CpG methylation (Li et al., 2005; Santoro, 2005; Santoro and Grummt, 2001; Santoro et al., 2002). By contrast, the 'active' euchromatic rDNA fraction that replicates in early S-phase represents rRNA genes competent for transcription whose activity is modulated according to the requirement of cell metabolism (Moss et al., 2007). Inheritance of silent rDNA chromatin is controlled by NoRC, the nucleolar remodeling complex comprising TIP5 and the ATPase SNF2h (Guetg et al., 2010; Santoro and Grummt, 2005; Santoro et al., 2002; Zhou et al., 2002). In mid-late S-phase, TIP5 binds to silent rRNA genes after the passage of the replication fork and recruits DNA methyltransferases and histone modifier enzymes to re-establish silent rDNA chromatin (Li et al., 2005; Santoro et al., 2002; Zhou et al., 2002). Knockdown of TIP5 impairs rDNA silencing and induces genome instability at the rDNA locus and at the nearby centric and pericentric sequences (Guetg et al., 2010; Santoro et al., 2009). NoRC function requires the association of TIP5 with the non-coding RNA (pRNA), a transcript originating from an RNA polymerase I (Pol I) promoter located 2kb upstream of the pre-rRNA transcription start site (Mayer et al., 2006; Santoro et al., 2010). pRNA is

synthesized by active rRNA genes during early S-phase and then processed during mid S-phase into a 250-300 nt fragment that matches the rDNA promoter sequences from -220 to +1 (Mayer et al., 2006; Santoro et al., 2010). Nucleolar retention of TIP5, rDNA methylation and silent histone modifications at rDNA depend on pRNA (Mayer et al., 2006). Importantly, a TIP5 mutant with impaired RNA binding activity (W531G,Y532A; $TIP5_{\Delta RNA}$) failed to establish rDNA heterochromatin. pRNA sequences from nucleotides -127 to -49 in mouse forms a conserved hairpin structure that is specifically recognized by the TIP5-TAM domain. Upon pRNA binding, TIP5 undergoes a conformational change that was proposed to facilitate the interaction with other proteins required for rDNA silencing (Mayer et al., 2008). We have now examined the mechanistic insights of NoRC-pRNA interaction that modulate recruitment of chromatin modifier enzymes to propagate rDNA heterochromatin during cell division.

Results

PARP1 associates with TIP5 and binds to silent rRNA genes

To dissect the mechanisms of NoRC function in rDNA heterochromatin formation, we identified TIP5 interaction partners in HEK293T cells line expressed HA-FLAG-TIP5 in combination with proteomics and immunoblot analysis. As shown in Figure 1A, we identified the poly(ADP-ribose) polymerases-1 (PARP1, also known as ARTD1 (Hottiger et al., 2010)) as TIP5-interacting protein. Lack of Pol I transcription factor UBF signal in TIP5-IP sample underscores the specificity of TIP5-PARP1 interaction. PARP1 is an enzyme possessing NAD^+-dependent catalytic activity to synthesize ADP-ribose (PAR) polymers bound to itself or to other proteins, including histones. Mapping of TIP5-PARP1 interaction domains by co-expression of myc-tagged PARP1 mutants and immunoprecipitation of HA-FLAG-TIP5 revealed that this association is mediated by the first N-terminal 341 aa of PARP1 (Figure 1B). This region comprises the two zinc-fingers

(FI/II), known to mediate binding to DNA. Although historically studied in the context of DNA genotoxic stress signaling, PARP1 has more recently been linked to the regulation of chromatin structure, transcription and chromosome organization (Hassa and Hottiger, 2008; Krishnakumar and Kraus, 2010). Accumulation of PARP1 in the nucleolus of interphase cells has been documented (Meder et al., 2005; Rancourt and Satoh, 2009) (Figure S1A). Consistent with this, a large fraction of PARP1 can be immuno-detected in purified nucleoli of NIH3T3 cells (Figure 1C). The role of PARP1 in the nucleolus remained so far unclear. To determine whether PARP1 associates with rRNA genes, we performed ChIP assays in NIH3T3 and HEK293T cells. Association of PARP1 with rDNA was detected at both promoter and coding regions (Figure 1D). A similar, but not identical rDNA binding profile was determined for UBF. As TIP5 binding is restricted to the promoter of silent rRNA genes (Figure S1B) (Santoro et al., 2002), we analyzed whether PARP1 associates either with the silent or active rDNA fraction. To test this, we performed a ChIP-chop assay (Santoro et al., 2002); that is, we isolated chromatin associated with PARP1 and UBF and monitored CpG methylation (the epigenetic mark characterizing the promoter of silent rRNA genes) of co-precipitated rDNA by methylation-sensitive restriction analysis. Consistent with previous results, UBF bound to unmethylated active rRNA genes (Figure 1E) (Santoro and Grummt, 2001; Santoro et al., 2002). In contrast, PARP1 was preferentially associated with the methylated, silent rDNA fraction. Taken together, the results indicated that PARP1 associates with TIP5, the subunit of the rDNA repressor NoRC complex, and binds to the promoter of silent rRNA genes.

Association of PARP1 with TIP5 is mediated by pRNA

To investigate the relationship between PARP1 and TIP5 in rDNA binding, we performed ChIP assays in HEK293T expressing shRNA-control, -*Parp1* and -*Tip5*

sequences (Figure 2A, Figure S2). In PARP1-depleted cells, the association of TIP5 with the rDNA promoter decreased when compared to shRNA-control cells. In TIP5-depleted cells, binding of PARP1 to the rDNA promoter decreased while the TIP5-dependent association with the coding region was inversely proportional to the distance from the rDNA promoter. These results suggest that recruitment of TIP5 and PARP1 to rDNA promoter is dependent on each other while the fraction of PARP1 bound to the second half of the coding region does not dependent on TIP5. The PARP1-mediated TIP5 binding to rDNA is supported by experiments showing that, after extraction of HEK293T chromatin with Triton X-100, the large majority of TIP5 in shRNA-control cells remained associated with chromatin (Mayer et al., 2006), whereas TIP5 was easily extracted in PARP1-depleted cells and enriched in the soluble fraction (Figure 2B). Previous results showed that nucleolar retention of TIP5 is disrupted after RNase A treatment (Mayer et al., 2006). Similarly to TIP5, treatment of cells with RNase A displaced PARP1 from nucleoli while the localization of UBF, as previously reported (Mayer et al., 2006), remained unaffected (Figure 2C). This result indicates that PARP1 nucleolar localization depends on RNA. To test whether PARP1 associates with pRNA, we measured pRNA content after immunoprecipitation of ectopic expressed HA-TIP5, -$TIP5_{\Delta RNA}$ or -PARP1 (Figure 2D). As expected, TIP5 but not $TIP5_{\Delta RNA}$ associated with pRNA. Importantly, although a large portion of PARP1 is involved in non-nucleolar activities, we detected a 2-fold enrichment of pRNA after PARP1-immunoprecipitation relative to control-IP, suggesting that PARP1 associates with NoRC/pRNA complex. Northwestern analysis determined that FI-II domains (*aa* 1-214) have the ability to associate with RNA (Figure 2H). This region coincides with the mapped TIP5-interaction domain of PARP1 shown in Figure 1B. A similar binding to RNA was also detected for the FIII (*aa* 215-373), while the region comprising the BRCT, WGR and the catalytic domain showed low or no

affinity for RNA. To analyze whether RNA mediates TIP5-PARP1 association, we performed FLAG immunoprecipitation of HA-FLAG-TIP5 and $TIP5_{\Delta RNA}$ in HEK293T cells. $TIP5_{\Delta RNA}$ did not associate with PARP1, suggesting that RNA, possibly pRNA, might mediate TIP5-PARP1 interaction (Figure 2E). Consistent with this, treatment of bead-bound TIP5-complexes with Ethidium bromide (EtBr), which by virtue of its ability to alter nucleic structure upon intercalation destabilizes protein associations mediated by DNA and RNA (Lai and Herr, 1992), strongly reduced TIP5-PARP1 association without affecting the interaction with Dnmt1, a known TIP5-interacting protein (Santoro et al., 2002) (Figure 2F). These results indicated that TIP5-PARP1 association is mediated by nucleic acids. As nuclear extracts were treated with DNase I and the DNA binding properties of $TIP5_{\Delta RNA}$ are not affected (Mayer et al., 2006), we reasoned that TIP5-PARP1 interaction is most probably mediated by RNA. To determine whether pRNA directly mediates TIP5-PARP1 association, we performed GST-pulldown assays using DNA/RNA-free purified recombinant GST-$TIP5_{1-598}$, containing the TAM domain, and His-$PARP1_{1-214}$, comprising FI-II domains. Binding reactions were performed in the absence or in the presence of different *in vitro* transcribed RNAs. In the absence of RNA, we did not detect association between TIP5 and PARP1, underscoring the role of RNA in this interaction (Figure 2G, lane 2). In contrast, in the presence of RNA, the TIP5-PARP1 interaction increased to different extents. The strongest signal corresponded to the reaction containing rRNA -232/-1 in sense orientation (lane 3). This RNA comprises the sequences (-127/-49) forming the conserved loop structure that is necessary for the interaction with TIP5 (Mayer et al., 2008). The antisense rRNA (-232/-1), the sense RNA (-127/-49) that lacks the sequence required to form the loop, and the control RNA (a 200 bases non-rRNA sequence) showed a very reduced or no ability to mediate TIP5-PARP1 binding when compared to the sense sequences -232/-1. These results indicate

that pRNA sequences, and specifically the region implicated in the formation of the loop structure (Mayer et al., 2008), directly mediate the TIP5-PARP1 association.

PARP1 establishes rDNA silencing via its ADP-ribosylation activity

To determine whether PARP1 is implicated in the formation of silent rDNA chromatin, we measured rRNA transcription and rDNA methylation levels in NIH3T3 cells selected for stable expression of shRNA-control, -*Tip5* or -*Parp1* sequences (Figure 3A-C). Consistent with previous results, rRNA synthesis increased and methylated rDNA levels reduced in the absence of TIP5 (Guetg et al., 2010; Santoro et al., 2009). Similar results were detected in PARP1 depleted HEK293T cells (Figure S3A), implying a role of PARP1 in rDNA silencing. Moreover, knockdown of PARP1 decreased the levels of H3K9me2 bound to rDNA, a histone mark associated with silent rDNA chromatin (Santoro et al., 2002) (Figure S3B). To support these data, we monitored rRNA transcription and rDNA promoter methylation levels in HEK293T cells overexpressing TIP5, PARP1 or $PARP1_{E988K}$ (a mutant lacking the ability to generate PAR polymers) (Rolli et al., 1997) (Figure 3D,E; S4A-C). Consistent with previous data, elevated levels of TIP5 induced repression of rRNA transcription (Figure 3D) that is mediated by *de novo* methylation of rRNA gene copies (Figure 3E) (Santoro and Grummt, 2005; Santoro et al., 2002). A similar transcriptional repression and increased methylated rDNA fraction was detected after PARP1 overexpression, indicating that elevated PARP1 levels promote *de novo* rDNA silencing. Taken together, the results show that PARP1 plays a central role in the formation of silent rDNA chromatin.

PARP1 is responsible for most of cellular PAR formation. During genotoxic stress, binding of PARP1 to DNA strand breaks catalyzes synthesis of PAR from NAD^+ and modifies many nuclear proteins, including itself and histones (Quenet et al., 2009). CoIP and ChIP assays revealed that the $PARP1_{E988}$ mutant that is unable to generate PAR

polymers binds to TIP5 and rDNA similarly to PARP1wt, indicating that PARP1-dependent parylation did not affect PARP1-TIP5 association and recruitment to rDNA (Figure 3F,G). Of note, the $PARP1_{E988K}$ mutant, was less efficient in repressing rRNA transcription and in methylating rDNA (Figure 3D,E; S4D). Consistent with this, the levels of H3K9me2 at rDNA were increased by overexpression of PARP1 but not by the $PARP1_{E988K}$ mutant (Figure S3C). These results suggest that PARP1-dependent parylation is implicated in the formation of silent rDNA chromatin.

Silent rDNA chromatin is substrate of parylation

The enzymatic activity of PARP1 is catalyzed by binding to DNA strand breaks (Krishnakumar and Kraus, 2010). The experiments described so far were performed in the absence of genotoxic stress and induced DNA breaks, raising the question how PARP1 activity can be promoted to establish rDNA silencing. To test whether RNA can activate PARP1, we measured PARP1 activity by monitoring automodification of an RNA/DNA free recombinant PARP1 in the presence of dsDNA or rRNAs (Figure 4A). As expected, no signal was detected in the absence of NAD^+ or nucleic acids while incubation with ds-oligomers and rDNA strongly stimulates PARP1 activity. Although at lower extent, rRNA stimulated PARP1 enzymatic activity but not in a sequence dependent manner. This result suggests that RNA might activate PARP1 when bound to TIP5. In support of this, incubation of the tandem affinity purified (TAP)-TIP5 complex with radiolabeled NAD^+ revealed automodification of PARP1, indicating that PARP1 bound to pRNA-TIP5 complex is enzymatically active (Figure 4B).

To determine whether components of the TIP5 complex are PARP1 substrates, we enhanced the parylation reaction by incubating bead-bound purified TAP-TIP5 or TAP-$TIP5_{\Delta RNA}$ complexes with recombinant PARP1 (rPARP1), radiolabeled NAD^+ and dsDNA (Figure 4C). After washing, bead-bound TAP-TIP5 or TAP-$TIP5_{\Delta RNA}$ complexes

were separated by gel-electrophoresis and labelled proteins were analyzed by gel-autoradiography. As shown in Figure 4C, rPARP1 efficiently binds to TIP5 complex and catalyzes its auto-modification and also parylation of TIP5 and other TIP5-interacting proteins. In contrast, rPARP1 incubated with $TIP5_{\Delta RNA}$ complex was unable to parylate $TIP5_{\Delta RNA}$ and displayed a reduced automodification activity that is probably due to its low binding efficiency to the $TIP5_{\Delta RNA}$ mutant. These results indicated that PARP1 parylates components of TIP5 complex but not $TIP5_{\Delta RNA}$ complex.

Next, we investigated whether silent rDNA chromatin is parylated. To date, the available antibodies recognizing PAR can detect only specific parylated proteins and they do not recognize some of the well-characterized targets of parylation, limiting their use for the detection of parylated proteins (Dani et al., 2009). Indeed, we did not detect specific signals, not even PARP1, by immunoblot of nucleolar extracts with 10H antibodies, that are generally used to identify long PAR polymers that form under genotoxic stress (Kawamitsu et al., 1984). This indicates that either nucleolar PAR levels were too low to be detected and/or polymers were too short. To overcome this technical limitation, we purified nucleolar parylated proteins that associate with the GST-macrodomain module *m*Af1521 (which binds parylated proteins potently and selectively) (Figure 4D, S5A) (Dani et al., 2009; Karras et al., 2005). This strategy has been recently used to identify parylated proteins in mammalian cells (Dani et al., 2009). As shown in Figure 4D, nucleolar histone H3 and PARP1 bind to *m*Af1521. This association is impaired in cells treated with the PAR inhibitor PJ34 or depleted of PARP1, indicating that nucleolar PARP1 is parylated and nucleolar chromatin is parylated by PARP1. Similarly, parylated nucleolar PARP1 and histone H3 were detected by aminophenyl boronate affinity chromatography, commonly used to purify parylated proteins (Figure S5B) (Adamietz et al., 1979; Okayama et al., 1978; Rosenthal et al., 2011). To

test whether parylation is a specific marker of silent rDNA chromatin, we measured incorporation of the NAD^+ analogue etheno-NAD^+ in nucleolar extracts (Figure S5C) using anti-ethenoadenosine antibodies. As expected, both histones and PARP1 are the major acceptor molecules of etheno moieties (Figure 4E). To analyze whether silent rDNA chromatin is a substrate for parylation, we measured binding of etheno-parylated proteins to rDNA by incubating nuclei with etheno-NAD^+ and, after crosslinking, by performing ChIP assays using anti-ethenoadenosine antibodies (Figure 4F; S5D). Etheno-parylated proteins were highly enriched at the rDNA promoter when compared to the promoter of the control gene IP10. The levels of etheno-PAR incorporation were reduced in cells depleted of PARP1 and TIP5, underscoring the role of PARP1 as the enzyme responsible for rDNA chromatin ADP-ribosylation and the role of TIP5 in recruiting PARP1 to the rDNA promoter. Notably, rDNA promoter sequences bound by etheno-parylated proteins were enriched in CpG methylation (Figure 4G). Taken together, the results indicated that nucleolar chromatin is parylated by PARP1 and that silent rDNA chromatin is a specific substrate of parylation.

PARP1 binds to silent rRNA genes after the passage of the replication fork

Previous data showed that the NoRC complex is implicated in the maintenance of silent rDNA chromatin that replicates in mid-late S-phase (Guetg et al., 2010; Li et al., 2005). Timing of synthesis and maturation of pRNA through S phase correlates with NoRC binding to silent rRNA genes during mid to late S-phase (Santoro et al., 2010), linking pRNA to propagation of silent rDNA chromatin during cell cycle. To determine whether binding of PARP1 to silent rRNA genes occurs during rDNA replication, we synchronized T24 cells in the cell cycle and incubated with BrdU 1 hour before sample collection (Figure 5A). The cell cycle distribution profile was determined by FACS analysis (Figure S6). Measurement of nascent rDNA isolated after immunoprecipitation

with anti-BrdU revealed the replication timing of active (early S-phase) and silent genes (mid S-phase) (Figure 5B; S7A). ChIP analysis indicated an increase in PARP1 binding to rDNA chromatin at mid-late S-phase and a decrease at later time of cell cycle (G2/M, M/G1) (Figure 5C, left panel; S7B). Anti-BrdU ChIP of PARP1-associated rDNA showed that PARP1 associates with rRNA genes that incorporated BrdU during mid S-phase, indicating that PARP1 binds to silent rRNA genes after the passage of the replication fork (Figure 5C, right panel). At this same time point (6 hours after the entry into S phase), we detected an enrichment of parylated nucleolar histone H3 when compared to the other times of S phase progression (Figure 5D; S8B). These results suggest that PARP1, once bound to newly synthesized rDNA (hour 6), resets the parylated state of new nucleolar chromatin. Parylation at this time point seems to be a transient event as indicated by the strong reduction of parylated histone H3 signal at end of S phase/G2 phase (Figure 5D; S8B). Coincident with the decreased binding of PARP1 to rDNA after conclusion of S phase, PARP1-TIP5 association was impaired from beginning of G2 phase (Figure 5E; S8A). Taken together, these results indicated that PARP1-TIP5 association is regulated during cell cycle and that PARP1 is recruited to silent rRNA genes after the passage of the replication fork. On the basis of our results, we propose that PARP1 and its associated enzymatic activity are implicated in the epigenetic inheritance of mid-late replicating silent rDNA chromatin (Figure 5F).

Discussion

The key finding of our work is that PARP1 is a critical component of the machinery that establishes and maintains silent rDNA chromatin during cell division. We found that PARP1: *i*- binds to TIP5 and that this interaction is mediated by pRNA; *ii*- associates with silent rRNA genes after the passage of the replication forks; *iii*- represses rRNA transcription and establishes silent rDNA chromatin *via* its ADP-ribosyltransferase activity (Figure 5F).

pRNA mediates TIP5-PARP1 association

Binding of PARP1 to silent rRNA genes after the passage of the replication fork (mid-late S-phase) correlates well with the timing of production of the mature pRNA and TIP5 association with rDNA (Li et al., 2005; Santoro et al., 2010). After replication of silent rRNA genes, mature pRNA might guide TIP5 to rDNA *via* the recently proposed triple helix formation (Schmitz et al., 2010) or by stabilizing TIP5 association with rDNA chromatin after recruitment mediated by the transcription terminator factor TTF-1, a known TIP5-interacting protein that binds to rDNA promoter in a sequence specific manner (Strohner et al., 2001). In both cases, the reported TIP5 conformational change induced upon binding to pRNA (Mayer et al., 2008) might favour the association of PARP1 and subsequent recruitment to newly synthesized rDNA. Similarly to pRNA, the lincRNA HOTAIR was recently shown to act as scaffold by providing binding surfaces to assemble the polycomb repressive complex 2 (PRC2) and the histone demethylase LSD1 at target genes (Tsai et al., 2010). The role of pRNA sequences in mediating the association of PARP1 with TIP5 and binding to newly synthesized silent rRNA copies strongly supports the idea that specific non-coding RNA can potentially direct complex patterns of chromatin states at specific genes in a spatially and temporally organized manner. Notably, TIP5-PARP1 interaction is impaired after completion of S phase.

These results suggest that this association is not only critical for a specific time window of cell cycle but also dynamic. Release of PARP1 from TIP5-pRNA might be modulated by posttranslational modifications. Recently, binding of Ezh2 to non-coding RNA HOTAIR and Xist has been reported to be upregulated when Ezh2 was phosporylated by cyclin-dependent kinase 1 (CDK1) (Kaneko et al., 2010). Many studies suggested that PARP1 activity can be regulated by phosphorylation involving several kinases that are parts of important regulatory pathways (Gagne et al., 2009). Whether and how a cell cycle regulated phosphorylation of PARP1 might influence the binding stability with TIP5-pRNA will be addressed in our future studies.

PARP1 is a critical component of the machinery that maintains silent rDNA chromatin during cell division

Our data unravelled a novel function of PARP1, that is to re-establish silent rDNA chromatin during DNA replication. Early studies determined a link between PARP1 and the DNA replication process. PARP1 was shown to co-localize with replication foci throughout S phase and to interact with several DNA replication proteins, many of which were poly ADP-ribosylated (Dantzer et al., 1998; Simbulan et al., 1993; Simbulan-Rosenthal et al., 1996; Sugimura et al., 2008). In addition, PARP activity was found to be enhanced in replicating cells (Lehmann et al., 1974), in the vicinity of replication forks (Jump et al., 1979) and in newly replicated chromatin (Anachkova et al., 1989). Notably, the role of PARP1 in DNA replication was mainly described in combination with DNA repair and recombination. For example, PARP1 was shown to collaborate with the repair protein Mre11 to promote replication fork restart after release from replication blocks. In line with this, PARP1 and PARP2 were described to be required for hydroxyurea-induced homologous recombination to promote cell survival after replication blocks (Bryant et al., 2009; Yang et al., 2004). Whether PARP1 plays a role for the inheritance

of specific chromatin states during DNA replication was so far not yet addressed. Binding of PARP1 to silent rRNA genes after the passage of the replication fork and its ability to establish rDNA silencing strongly suggest a critical role in the inheritance of silent rDNA chromatin during cell division. In support of this, recent results identified PARP1 as SMARCAD1 interacting protein in several human cell lines (Rowbotham et al., 2011). SMARCAD1 is recruited to sites of DNA replication and ensures that silenced loci, such as pericentric heterochromatin, are correctly re-established. Although the role of PARP1 in the maintenance of pericentric heterochromatin mediated by SMARCAD1 was not explored by this study, we considered that this hypothesis could not be excluded. Recently, we showed that TIP5 binds to major and minor satellite DNA and that TIP5 knockdown impairs heterochromatin formation at these repeats and induces genome instability (Guetg et al., 2010). Lack of PARP1 is associated with severe chromosomal instability, characterized by increased frequencies of chromosome fusions and aneuploidy (d'Adda di Fagagna et al., 1999). Interestingly, we found that PARP1 binds to mouse centric repeats and human alpha satellite DNA (Figure S7B, S9A,B) and that depletion or overexpression of PARP1 affects the H3K9me2 levels at the alpha satellite DNA (Figure S3). Whether the function of PARP1 in the propagation of silent rDNA chromatin can be linked to the maintenance of centric- pericentric heterochromatin it will be analyzed in our future work.

The enzymatic activity of PARP1 is required for rDNA silencing

Many evidences indicated a paradoxical dual contribution of PARP1 in transcription regulation. PARP1 was implicated in the formation of chromatin structures that are permissive to transcription. In MCF-7 breast cancer cells, PARP-1 localizes to the promoters of almost all actively transcribed genes and acts to exclude linker histone H1 from a subset of PARP1-stimulated promoters (Kim et al., 2004; Krishnakumar et al.,

2008). On the other hand, our study showed that PARP1 preferentially binds to the promoter of silent rRNA genes and participates in the establishment of rDNA silencing. Similarly, PARP1 was reported to bind to constitutive heterochromatin regions, including the centromeres (Kanai et al., 2003) and telomeres (Beneke et al., 2008). In *Drosophila*, genetic studies indicated that PARP1 is necessary to organize the chromatin structure of nucleoli and heterochromatin domains and to silence retrotransposable elements (Kotova et al., 2010; Tulin et al., 2002). The enzymatic activity of PARP1 was proposed as the switch event that might distinguish between a PARP1 with co-repressor and co-activator function (Ji and Tulin, 2010). The ability to disrupt chromatin structure by parylating histones and destabilizing nucleosomes was one of the earliest functional effects of PARP1 to be characterized (Huletsky et al., 1989; Kim et al., 2004; Mathis and Althaus, 1987; Messner and Hottiger, 2011; Poirier et al., 1982; Wacker et al., 2007). The role of parylation in decondensing chromatin finds its best example in the rapid accumulation of PAR at heat shock loci in response to heat shock in *Drosophila* (Tulin and Spradling, 2003). dPARP is required for heat shock-induced ‘‘puffing’’ (i.e., chromatin decondensation) and knockdown of dPARP or treatment with a PARP inhibitor prevents heat shock-induced nucleosome loss and enhanced transcription at the Hsp70 gene (Petesch and Lis, 2008). However, examples exist where PARP1, when acting as co-activator, does not require its enzymatic activity (Hassa and Hottiger, 2002; Kraus and Lis, 2003; Pavri et al., 2005). Our data pointed out that the enzymatic activity of PARP1 is not only limited to processes where PARP1 acts as co-activator. We showed that PARP1-mediated parylation affects formation of rDNA silencing and that silent rDNA chromatin is substrate for parylation. These results are consistent with previous studies showing that many of the *Drosophila* modified proteins detectable with anti-PAR antibodies were particularly enriched in nucleoli and in the heterochromatic chromocenter regions (Tulin et al., 2002). Our data indicated that nucleolar histones are

parylated by PARP1 and that PARP1 can parylate itself and other components of the NoRC complex, including TIP5. The observed increase in nucleolar histone parylation, at the time when rRNA genes are replicated and bound by PARP1, strongly suggests a functional link between parylation and the re-establishment of silent rDNA chromatin. Consistent with this, $PARP1_{E988K}$ (a mutant lacking the ability to generate PAR polymers) was less efficient in repressing rRNA transcription and in establishing silent rDNA chromatin. Taken together, the results suggest that the propagation of silent marks at the rDNA locus requires PARP1 activity. Notably, our results showed that RNA has the ability to activate PARP1. Thus, pRNA might not only mediate the association of PARP1 with TIP5 but also modulate the enzymatic activity of PARP1.

There are many possibilities by which parylation can act to establish silent rDNA chromatin. PARP1 could covalently modify another protein to activate the rDNA silencing process. We showed that components of the NoRC complex, including TIP5, are parylated by PARP1. Recent progresses in PAR-mass spectrometry (Messner et al., 2010) will allow determining whether and how parylated NoRC complex affects the formation of rDNA heterochromatin. Alternatively, histone parylation might serve to destabilize nucleosomes to gain accessibility to the action of DNA methyltransferases and/or of histone modifying enzymes. Moreover, parylation of histones might facilitate the deposition of silent histone modifications by docking chromatin enzymes.

The identification of PARP1 and parylation as regulators of rDNA silencing adds a further layer of complexity in the readout of PAR signalling. Our assays did not detect formation of long PAR polymers, typically forming upon genotoxic signalling and generation of DNA strand breaks or in “puff” formation. If the length or the structure of the generated PARs might represent a critical mark that distinguishes PARP1 as coactivator or corepressor remains yet to be elucidated. The contribution of PARP1 in both activating and repressing transcription can be also appreciated in the nucleolus

compartment. In addition to our studies showing binding of PARP1 to the rDNA repressor TIP5, previous results identified the association of PARP1 with B23 and nucleolin, nucleolar proteins involved in several processes including rDNA transcription and elongation, ribosome assembly and rRNA processing (Leitinger and Wesierska-Gadek, 1993; Meder et al., 2005). Based on these results and the fact that a fraction of PARP1 that associates with the second half of the rDNA coding region is less dependent on TIP5 when compared to the PARP1 fraction bound to the promoter (Fig. 2A), we predict that PARP1 might play additional roles in regulating nucleolar activities. Generation of antibodies specific to parylated histones and recent advances in PAR-mass spectrometry (Messner et al., 2010) will in the next future allow deciphering how the code of parylated histones or other chromatin and transcription regulators is mechanistically interpreted.

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Figure legends

Figure 1

PARP1 associates with TIP5 and silent methylated rRNA genes. (**A**) FLAG-immunoprecipitation from HEK293T cells expressing FLAG-HA-TIP5. Immunoblots show association of TIP5 with SNF2h (a component of NoRC complex) and PARP1 but not with the rDNA transcription factor UBF. (**B**) TIP5 interacts with PARP1-zinc-finger FI/II domains. FLAG-immunoprecipitation of HEK293T cells co-transfected with myc-$PARP1_{1-341}$ and myc-$PARP1_{341-1014}$ expressing plasmids in the presence or absence of vectors expressing HA-FLAG-TIP5. Co-precipitated proteins were visualized using HA- and Myc-antibodies. The schema represents the PARP1 domains that were expressed for the interaction studies. (**C**) PARP1 is present in the nucleoli. Identical NIH3T3 cell equivalents of purified nucleoli and nuclei were analyzed for PARP1, UBF and Pol II enrichment with the corresponding antibodies. The purity of nucleoli was assessed by the lack of Pol II signals. (**D**) PARP1 associates with rRNA genes. PARP1 and UBF occupancy at the rRNA gene in HEK293T and NIH3T3 cells was analyzed by ChIP. Data are represented as bound/input values normalized to the occupancy at the rDNA promoter (+1). (**E**) PARP1 is associated with the promoter of methylated silent rRNA genes. ChIP-chop analysis showing meCpG content of rDNA promoter sequences from total rDNA and of chromatin immunoprecipitated with PARP1 and UBF antibodies. CpG methylation was assayed by digestion with HpaII (NIH3T3) or SmaI (HEK293T). The bars indicate the relative level of methylated silent rDNA (HpaII/SmaI-resistant, black) compared to unmethylated, active rDNA (light) measured by qPCR. Error bars indicate the s.d. of three independent experiments.

Figure 2

PARP1-TIP5 association is mediated by pRNA. (**A**) ChIP showing occupancy at rDNA regions in HEK293T cells after depletion of PARP1 and TIP5 by shRNA. Values (bound/input) are normalized relative to rDNA occupancy of cells expressing shRNA-control sequences. IL6 represents a control gene that associates with PARP1 but is not regulated by TIP5. Error bars indicate the s.d. of two independent experiments. ***, $P < 0.001$ vs. Control. (**B**) PARP1 is required for the association of TIP5 with chromatin. Identical cell equivalents from chromatin bound (Chr.) and soluble (Sol.) fractions of HEK293T cells expressing shRNA-control and *Parp1* sequences were visualized with anti-TIP5 and PARP1 antibodies. Coomassie staining of the membrane showed equivalent protein loading and efficiency of chromatin extraction. Relative values of chromatin-bound and soluble TIP5 are indicated (two independent experiments). (**C**) PARP1 nucleolar localization is sensitive to RNase A treatment. Permeabilized NIH3T3 cells were treated with RNase A and localization of PARP1 and UBF was visualized by immunofluorescence. (**D**) PARP1 associates with pRNA. qRT-PCR. RIP assay monitoring levels of pRNA associated with immunoprecipitated HA-TIP5, $TIP5_{\Delta RNA}$ and PARP1 in HEK293T cells. pRNA levels were normalized to 28S rRNA and IP from cells transfected with empty vector (Contr.). Error bars indicate the s.d. of three independent experiments. **, $P < 0.01$ vs. $TIP5_{\Delta RNA}$. (**E**) RNA mediates association of PARP1 with TIP5. Flag-immunoprecipitation of HEK293T cells expressing HA-FLAG-TIP5 and -$TIP5_{\Delta RNA}$. (**F**) Bead-bound FLAG-TIP5 immunoprecipitates from transfected HEK293T cells were incubated with or without EtBr (10 μg/ml*)* and washed again. Co-precipitated proteins were visualized on immunoblots with anti-HA, DNMT1 and PARP1 antibodies. The data show one representative experiment out of two independent experiments. (**G**)

pRNA mediates association of PARP1 with TIP5. Upper panel. Schema representing mouse rDNA promoter sequences, including the region -127 to -49 forming the conserved hairpin structure that is specifically recognized by TIP5**.** *In vitro* transcribed rRNAs used for this assay are shown below (rRNA -232/-140, -232/-1; 200 bases non-ribosomal RNA sequences, contr.). Sense (S) and antisense (AS) orientation are indicated. Lower panel. RNA/DNA free purified recombinant bacterial expressed GST-$TIP5_{1-598}$ and His-$PARP1_{1-214}$ were incubated with the indicated 5 pmoles renaturated RNAs and GST-pulldown proteins were visualized by immunoblots using anti-GST and anti-His antibodies. (**H**) Northwestern. The schema represents PARP1 proteins that were analyzed for RNA binding. Similar amounts of the membrane-bound recombinant PARP1 proteins were incubated with radiolabeled pRNA sequences (-232/-1) and bound RNA was visualized by autoradiography. The Coomassie staining shows the amounts of proteins used in this assay.

Figure 3

PARP1 mediates rDNA silencing. (**A**) Immunoblot (upper panels) and mRNA levels (lower panel) showing depletion of TIP5 and PARP1 in NIH3T3 cells. mRNA levels were normalized against rsp12 mRNA. (**B**) 45S pre-rRNA levels (**C**) and meCpG content at the rDNA promoter in NIH3T3-shRNA-control, -Tip5 and -PARP1 cells. rRNA levels were measured by qRT-PCR and normalized to rsp12 mRNA and to shRNA-control cells. meCpG content was measured after digestion with HpaII. Error bars indicate the s.d. of two independent experiments. $*$, $P < 0.05$ vs. Control. (**D**) 45S pre-rRNA levels of HEK293T cells overexpressing HA-TIP5, -PARP1 and -$PARP1_{E988}$. Values were measured by qRT-PCR as described above. Error bars indicate the s.d. of four independent experiments. $*$, $P < 0.05$ vs. PARP1wt. (**E**) The same cells were analyzed

for meCpG content of rDNA promoter by SmaI digestion. The Sma I resistant methylated rDNA fraction was measured by qPCR. Error bars indicate the s.d. of four independent experiments. **, $P < 0.01$ vs. PARP1wt. PARP1-TIP5 association (**F**) and PARP1 recruitment to rDNA (**G**) are independent of PARP1-activity. (**F**) Flag-immunoprecipitation of HEK293T cells expressing HA-PARP1 or HA-PARP1$_{E988K}$ with or without HA-FLAG-TIP5. Immunoprecipitates were detected with anti-HA antibodies. (**G**) ChIP analysis of HA-PARP1 and HA-PARP1$_{E988K}$ in HEK293T cells depleted of endogenous PARP1 by shRNA. Analysis was performed with anti-HA antibodies. Cells transfected with empty vectors were used as control. Error bars indicate the s.d. of three independent experiments.

Figure 4

Silent rDNA chromatin is parylated. (**A**) RNA stimulates PARP1 enzymatic activity. Automodification of DNA/RNA-free PARP1 incubated with radiolabeled NAD^+ in the presence or absence of the indicated amounts of DNAs and rRNAs. (**B**) TIP5-associated PARP1 is enzymatically active. Tandem affinity purified bead-bound TAP and TAP-TIP5 complex from HEK293T cells were incubated with radiolabeled NAD^+. Automodified PARP1 was detected by autoradiography. As control, recombinant (r)PARP1 was included in the analysis. (**C**) TIP5 is substrate for PARP1-parylation. Bead-bound purified TAP, TAP-TIP5 and -TIP5$_{\Delta RNA}$ complexes from HEK293T cells, were incubated with recombinant (r)PARP1, radiolabeled NAD^+ and ds-oligonucleotides. After washing, bead-bound parylated peptides were visualized by SDS-page gel autoradiography. Silver staining showed equivalent TAP-TIP5 and -TIP5$_{\Delta RNA}$ amounts used for the assay. (**D**) Parylated proteins from purified nucleolar extracts of HEK293T cells, treated with or without the PARP inhibitor PJ34 and expressing shRNA-control and PARP1 sequences,

were pulled down with GST or GST-mAf1521 domain. PARP1 and histone H3 were visualized by immunoblots. (**E**) Purified nucleoli from NIH3T3 cells were incubated with and without etheno-NAD^+. Histone and PARP1 incorporation of etheno moieties was monitored with anti-ethenoadenosine antibodies. Histone H3 and PARP1 were detected with the corresponding antibodies. (**F**) ChIP assay showing incorporation of etheno-moieties at rDNA and IP10 promoters in HEK293T cells expressing shRNA-Control, -*Parp1* and -*Tip5* sequences. Values (bound/input) are normalized to the association of etheno with the rDNA promoter of control cells. (**G**) Methylated silent rDNA chromatin is substrate for parylation. Anti-etheno ChIP samples were subjected to ChIP-chop analysis. The bars indicate the relative level of SmaI-resistant, methylated silent genes (black) and unmethylated, active genes (light). Error bars indicate the s.d. of three independent experiments.

Figure 5

PARP1 binds to newly replicating silent rRNA genes. (**A**) Protocol for synchronization of the cell cycle and BrdU labeling in T24 cells. (**B**) Replication timing of rRNA genes in T24 cells. Nascent DNA from synchronized cells was immunoprecipitated using anti-BrdU antibodies. To calibrate for DNA recovery during IP, BrdU-labelled *E. coli* DNA was added to the reactions. Nascent DNA was measured by qPCR. The values represent the amounts of immunoprecipitated DNA normalized to inputs and to the amounts of BrdU-labelled β-lactamase gene. (**C**) Left panel. ChIP (1^{st}) showing binding of PARP1 to rDNA promoter during cell cycle. The data are presented as the amounts of bound normalized to input and IgG control. Right panel. ChIP (2^{nd}) showing association of PARP1 with rDNA after replication in mid S-phase. PARP1 associated rDNA was immunoprecipated with anti-BrdU antibodies. The values represent the amounts of immunoprecipitated DNA normalized to the amounts of BrdU-labelled β-lactamase gene, input and IgG

control. (**D**) Enrichment of parylated nucleolar histone H3 during mid S-phase. Aminophenyl boronate (PB) affinity chromatography of nucleolar extracts purified from T24 cells during the indicated times of S phase progression. (**E**) PARP1 and TIP5 associate during S phase. Dox-inducible HA-FLAG-TIP5/HEK293T cells were synchronized at G1/S, collected every 2 h post-release and FLAG-immunoprecitated samples were analyzed by immunoblot using anti-HA, -Snf2h and -PARP1 antibodies. (**F**) Model showing the inheritance of silent rDNA chromatin mediated by TIP5, pRNA and PARP1. After the passage of the replication fork in mid S-phase, TIP5-pRNA-PARP1 complex binds to nascent rRNA genes. pRNA mediates association of TIP5 and PARP1 and activates the enzymatic activity of PARP1 to parylate PARP1 itself, TIP5 or histones. PARP1 enzymatic activity facilitates formation of silent rDNA chromatin and transcriptional silencing.

Supporting online material

Supplemental Figure S1

(**A**) Immunofluorescence analysis of PARP1 in U2OS cells showing nucleoplasmatic and nucleolar localization. (**B**) ChIP analysis in HEK293T cells showing the specific association of TIP5 with the rDNA promoter and its exclusion from the coding region. Data are represented as bound/input values normalized to the occupancy at the rDNA promoter (+1).

Supplementary Figure S2

PARP1 and TIP5 expression levels in HEK293T cells after depletion of TIP5 or PARP1 by shRNA. The data showed knockdown efficiency of the experiments of Fig. 2A and that PARP1 levels are not affected by TIP5 knockdown and *vice versa* (see also Fig. 3A for NIH3T3 cells). Data are normalized against GAPDH mRNA levels. Error bars

indicate the s.d. of the two independent experiments shown in Figure 2A.

Supplementary Figure S3

PARP1 and its parylation-activity are implicated in the formation of silent rDNA chromatin. (**A**) PARP1 mRNA and 45S pre-rRNA levels in HEK293T cells expressing shRNA-control and *Parp1* sequences. (**B**) ChIP showing that the levels of H3K9me2 decreased at the rDNA promoter and alpha satellite sequences but not at the IL6 promoter region in HEK293T cells depleted of PARP1. (**C**) ChIP showing that overexpression of PARP1 increased the levels of H3K9me2 at the rDNA promoter and alpha satellite sequences but not at the IL6 promoter region. Overexpression of the mutant $PARP1_{E988K}$ did not affect the levels of H3K9me2 at the rDNA promoter. In contrast H3K9me2 associated alpha satellite DNA were similar in cells that express either PARP1 or the mutant $PARP1_{E988K}$.

Supplementary Figure S4

Overexpression of TIP5 and PARP1 did not affect the endogenous levels of TIP5 and PARP1. Western blots showing that ectopically expressed HA-FLAG-TIP5 (**A**) and HA-PARP1 (**B**) do not affect endogenous PARP1 and TIP5 levels, respectively. (**C**) Ectopically expression of HA-PARP1 does not affect NoRC complex (TIP5-SNf2h). FLAG-IP from HEK293T cells expressing HA-FLAG-TIP5 with or without HA-PARP1. HA-FLAG-TIP5 and HA-PARP1 were detected with anti-HA antibodies. SNF2h with anti-SNF2h antibodies. (**D**) Western blot showing similar expression levels of HA-PARP1 and HA-PARP1 E988K . UBF immunoblots serve as normalization control.

Supplementary Figure S5

(**A**) Purity of the nucleoli used in Figure 4D was assessed by comparing the levels of Polymerase II between nuclear and nucleoli extracts. The levels of nucleolar UBF serve

to compare similar cell equivalents. (**B**) Nucleolar histone H3 is parylated. Aminophenyl boronate (PB) affinity chromatography of nucleolar extracts from HEK293T cells treated with or without the PARP inhibitor PJ34. (**C**) Purity of the nucleoli used in Figure 5E was assessed by comparing the levels of Polymerase II between nuclear and nucleoli extracts. The levels of nucleolar UBF serve to compare similar cell equivalents. (**D**) ChIP assay showing the specificity of the etheno-ChIP method. Prior formaldehyde crosslinking, nuclei were incubated without Etheno-NAD^+, with etheno-NAD^+ and the PARP inhibitor PJ34 or only with Etheno-NAD^+. Values (bound/input) are normalized to the association of etheno with the rDNA promoter in cells treated with Etheno-NAD^+.

Supplementary Figure S6

Profile of cell cycle progression of T24 cells. T24 cells were arrested in G0 by contact inhibition. After at least 2-3 days of confluence, the cells were split by seeding multiple 100-mm dishes at a concentration of ≈ $3 \cdot 10^6$ cells per dish. After 14 hours cells reached G1/S (here referred as t=0h). Values (%) of cells in G1, S and G2/M phases are shown.

Supplementary Figure S7

(A) Replication timing of alpha satellite repeats in T24 cells. Nascent DNA from synchronized cells was immunoprecipitated using anti-BrdU antibodies. Replication timing of rRNA repeats is shown in Figure 5B. To calibrate for DNA recovery during IP, BrdU-labelled *E. coli* DNA was added to the reactions. Nascent DNA was measured by qPCR. The values represent the amounts of immunoprecipitated DNA normalized to inputs and to the amounts of BrdU-labelled β-lactamase gene. (**B**) ChIP showing binding of PARP1 to alpha satellite repeats during cell cycle. Association of PARP1 with rRNA genes is shown in Figure 5C. The data are presented as the amounts of bound normalized to input and IgG control.

Supplementary Figure S8

(**A**) Profile of cell cycle progression of T-Rex^{TM}-293 stable cell line that expresses HA-Flag-TIP5 under doxycyclin induction (see Figure 5E) and (**B**) of T24 cells (see Figure 5D).

Supplementary Figure S9

PARP1 associates with centric and pericentric repeats. (**A**) ChIP showing the association of PARP1 with the mouse rDNA promoter, minor and major satellites in NIH3T3 cells. (**B**) ChIP showing the association of PARP1 with the human rDNA promoter and alpha satellite repeats. Data are represented as bound/input values normalized to the occupancy at the rDNA promoter (+1).

Methods

Plasmid

The following plasmids were used in this study: pLTR-PPT-RRE-$CMV_{promoter}$-HA-FLAG-TIP5-PGK-puro; pLTR-PPT-RRE-$CMV_{promoter}$-TAP-TIP5; pcDNA-5/FRT/TO-HA-FLAG-TIP5; pGEX-$TIP5_{1-598}$; pcDNA-myc-$PARP1_{1-341}$; pcDNA-myc-$PARP1_{341-1014}$; pcDNA-HA-PARP1; pcDNA-HA-$PARP1_{E988K}$; pLTR-PPT-RRE-H1 promoter-TetO2-sh/$hTIP5_{nt244-264}$-PGK-Neo; pLTR-PPT-RRE-$H1_{promoter}$-TetO2-shControl-PGK-Neo; pLTR-PPT-RRE-$H1_{promoter}$-TetO2-sh/$hPARP1_{nt3130-3148}$-PGK-Neo; pLTR-PPT-RRE-$H1_{promoter}$-TetO2-sh/$mPARP1_{nt916-936}$-PGK-Neo. The indicated pRNA and control sequences were cloned by PCR into pCR2.1-TOPO vectors.

Cell lines and cell cycle synchronization

T-Rex^{TM}-293 stable cell line that expresses HA-Flag-TIP5 under doxycyclin induction was generated according to the manufacture's protocol (Invitrogen). Expression was induced with 1µg/ml doxycyclin (Sigma) for 24 hours before harvesting. For synchronization, cells were collected 2 hours post-release from a thymidine (2 mM) / mimosine (400 µM) double-block. NIH3T3 cells were selected for stable expression for 10 days after transduction with retroviruses expressing shRNA-control, -*Tip5* or -*Parp1* sequences. T24 bladder tumor cells were arrested in G0 by contact inhibition (Jin et al., 1997). After 2-3 days of confluence, the cells were split by seeding multiple 100-mm dishes at a concentration of ≈ $3 \cdot 10^6$ cells per dish. After 14 hours, cells reached G1/S phase (here referred to as t=0h). Cell cycle synchronization and progression was confirmed by flow cytometry (FACS).

Immunoprecipitation

Immunoprecipitations were performed by transfection of HEK293T cells with expression

vectors encoding the respective proteins (HA-FLAG-TIP5, HA-FLAG-TIP5$_{\Delta RNA}$, Myc-PARP1$_{1-341}$ and Myc-PARP1$_{341-1014}$). After 48 h, we lysed the cells in lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% Glycerol, 0.1% NP-40, proteinase inhibitor cocktail (Roche)), followed by DNase I treatment for 30 min at 4 °C. The cleared lysate was subjected to immunoprecipitation overnigth at 4 °C using immobilized antibody against FLAG or HA (ANTI-FLAG M2 or ANTI-HA affinity gel, Sigma). Precipitates were washed three times with wash buffer (20 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% Glycerol, 0.1% Tween, 0.1 mM PMSF), separated on a 6% SDS-polyacrylamide gel and analyzed by immunoblot. For RIP experiments, one half of the bead-bound immunoprecipitates were analyzed for proteins levels by immunoblot using anti-HA antibodies. The other half was double-purified for RNA isolation (Triazol, Invitrogen), including DNase I treatment (Fermentas, 1 U, 20 min at 37 °C) according to the manufacture's protocol. pRNA and control RNA (*28S* rRNA) levels were quantified by qRT-PCR. Amplification of samples without reverse transcriptase assured absence of DNA (data not shown).

Immunofluorescence

Cells grown on coverslips were permeabilized with 0.05% Triton X- 100 in 20mMTris-HCl (pH 7.4), 5mMMgCl2, 0.5mMEDTA, and 25% glycerol; washed with PBS; and, when indicated, treated for 7 min with RNase A (1mg/ml). After washing, cells were fixed with cold methanol (7 min) and stained with anti-PARP1 and anti-UBF antibodies, and immunofluorescent images were digitally recorded.

Chromatin immunoprecipitation

ChIP analysis was performed as previously described (Santoro *et al*, 2002). CpG methylation was assayed by digestion with HpaII (NIH3T3) or SmaI (HEK293T). Values

were calculated by qPCR using primer pairs that flank the restriction sites on the promoter and primers amplifying neighboring sequences lacking HpaII/SmaI sites. Anti-BrdU IP and ChIP were performed on synchronized cells that were pulse labelled (1 hour) with 30 μM 5'-BrdU before sample collection (Guetg et al., 2010). Nascent DNA was isolated, purified and measured by qPCR. To calibrate DNA recovery, BrdU-labeled *E.coli* DNA was added to the reactions prior immuno-precipitations. Values were normalized to the amounts of β-lactamase calculated by qPCR.

Expression and purification of recombinant proteins

GST-tagged $TIP5_{1-598}$ was expressed in *Escherichia coli* BL21 cells and purified on GST beads (Glutathione Sepharose 4B, GE Healthcare). Recombinant PARP1 and $PARP_{1-214}$ were expressed as C-terminal His-tagged proteins in insect cells and purified by one step affinity chromatography using ProBond resin according to the manufacturer's recommendations (Invitrogen). Expression and purification of the recombinant proteins were analyzed by SDS–PAGE followed by coomassie staining. When indicated, purified recombinant proteins were incubated with 5 ng/ml RNase A and DNase I for 30 min at 4 °C, followed by incubation with 20U RNase Inhibitor (Applied Biosystem). RNase A inactivation was monitored by comparing RNAs after incubation in the presence and absence of purified RNA/DNA-free recombinant GST-$TIP5_{1-598}$ and his- $PARP1_{1-214}$ proteins.

***In vitro* transcription**

rRNA and control RNA (hKCNA, sequences from +1 to +237) were synthesized using T7 polymerase and as substrate linearized vectors containing the indicated sequences. After treatment with DNase I, transcripts were double purified using TRIzol reagent

(Invitrogen) according to the manufacture's protocol. Prior to experimental use, the RNA was renaturated in 1x REN buffer (10mM Tris-HCl, pH 7.5, 200mM NaCl, 12mM $MgCl_2$, 0.4mM EDTA), heated 5 min at 70 °C, and slow-cooled to room temperature.

GST pull-down assays

GST-TIP_{1-598} was incubated with 5 µg His-$PARP1_{1-214}$ in presence of 5 pmol of the indicated renaturated RNA for 4 hours at 4 °C. After three times washing with EBC-Wash buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 5 mM DTT, 1 mM PMSF) GST-TIP5 bound proteins were separated by SDS–PAGE and analyzed by western blotting. Nucleolar parylated proteins were purified by incubating nucleolar extracts with GST-mAf1521 as previously described (Dani et al., 2009).

ADP-ribosylation assay

^{32}P-NAD^+ ADP-ribosylation was performed as previously described (Messner et al., 2010). TAP and TAP-TIP5 complexes were purified on IgG Sepharose 6 Fast Flow beads (GE Healthcare) and incubated with 100 nM radiolabeled NAD^+ (^{32}P-NAD^+) with or without 10 pmol PARP1 and 5 pmol ds-oligonucleotides. Reaction was carried out at 30 °C for 10 min in ADP-ribosylation buffer (50 mM Tris–HCl, pH 8.0, 4 mM $MgCl_2$, 250 µM DTT, 20 mM NaCl, protease inhibitor cocktail). After the *in vitro* ADP-ribosylation, bead-bound proteins were washed three times with wash buffer, resolved on a 6% SDS-polyacrylamide gel and visualized by exposure to X-ray films. 1,N^6-etheno-NAD (ε-NAD) was purchased from Sigma. Nuclei and nucleoli, prepared according to the protocol http://www.lamondlab.com/f7nucleolarprotocol.htm, were resuspended in a buffer containing 53% buffer 1 (10 mM Tris-HCl (pH 7.8), 4 mM $MgCl_2$, 1 mM EDTA, and 30 mM β-mercaptoethanol), 33% buffer 2 (100 mM Tris-HCl, pH 7.8, and 120 mM $MgCl_2$)

with or without 400 mM e-NAD, that is the approximate nuclear concentration of NAD^+ *in vivo*. Samples were incubated 30 min at 37°C and, when indicated, crosslinked with 1% formaldehyde. ADP-ribosylated proteins were purified using aminophenyl boronate affinity chromatography. 15 μL of slurry Prosep-PB resin (Millipore) were washed with 1ml binding/wash buffer (250 mM ammonium acetate, 50 mM $MgCl_2$, 100 mM Tris-HCl, 500 mM NaCl (pH 8.8)) and incubated with nucleolar extracts. After two washes with binding/wash buffer, beads were washed twice with 50mM ammonium acetate pH 8.8 and resuspended in SDS loading buffer. After boiling beads were run on an SDS gel and enriched proteins were detected by western blot analysis.

Northwestern

His-tagged recombinant PARP1 proteins were affinity-purified, separated by SDS-PAGE and transferred to nitrocellulose filters. Proteins were renatured in buffer containing 10 mM Tris-HCl [pH 6.8], 25 mM NaCl, 1 mM EDTA, 0.04% BSA, 0.04% NP40. ^{32}P-labeled pRNA (-232/-1) was added, the membrane was incubated for 2 h at room temperature, washed three times, and bound RNA was monitored by autoradiography.

Antibodies

Anti-TIP5 (CS-090-100) antibodies were from Diagenode. Anti-Dnmt1, anti-histone H3 and anti-ethenoadenosine were from Abcam. Anti-HA, anti-Snf2h, anti-PARP1, anti-Polymerase II, anti-c-Myc, anti-GST and anti-UBF antibodies were from Santa Cruz. Anti-His antibodies were obtained from Qiagen.

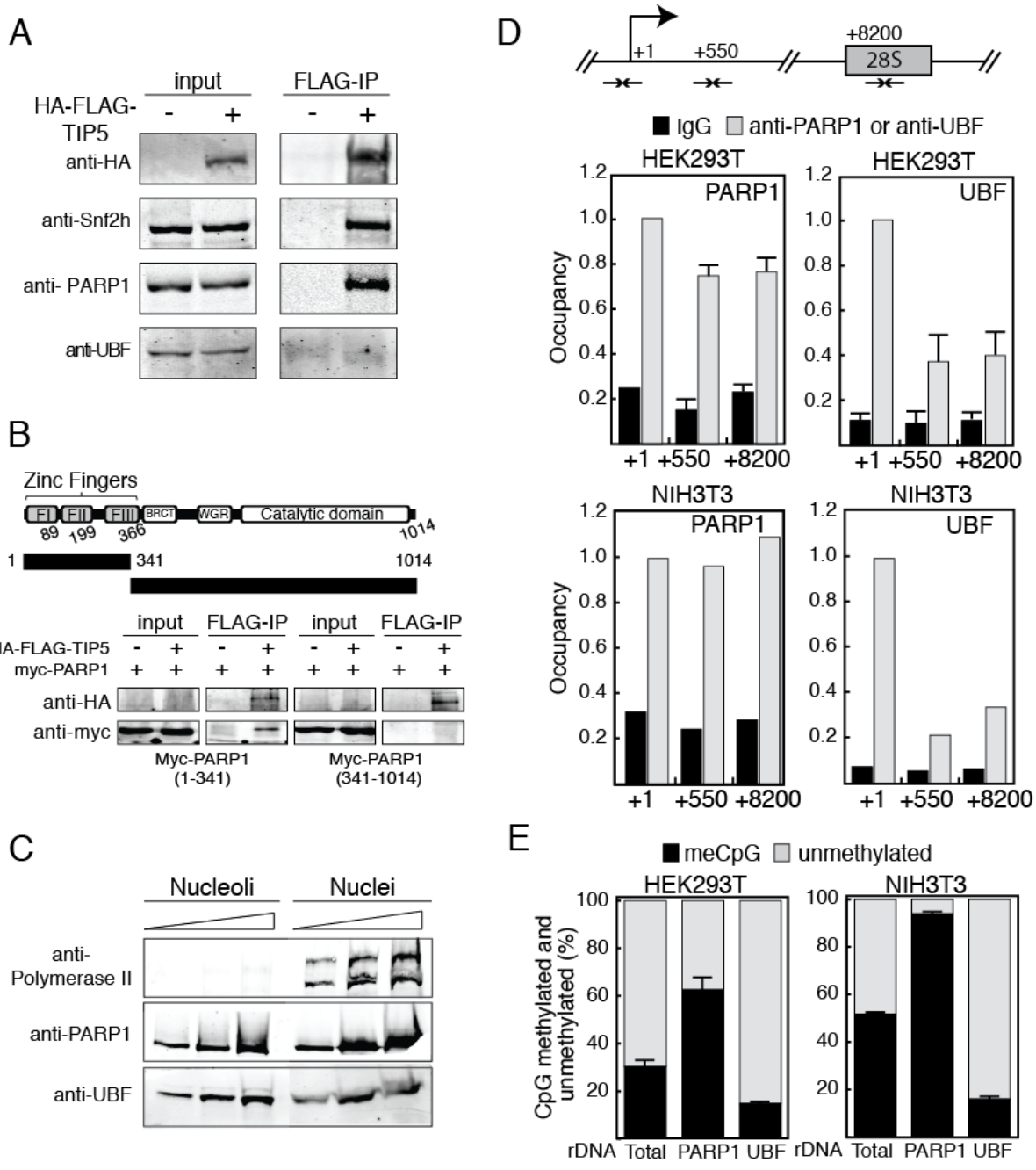

A
input
FLAG-IP
HA-FLAG-TIP5
- + - +
anti-HA
anti-Snf2h
anti- PARP1
anti-UBF
B
Zinc Fingers
FI FII FIII BRCT WGR Catalytic domain
89 199 366 1014
1 341 1014
input FLAG-IP input FLAG-IP
HA-FLAG-TIP5 - + - + - + - +
myc-PARP1 + + + + + + + +
anti-HA
anti-myc
Myc-PARP1 (1-341)
Myc-PARP1 (341-1014)
C
Nucleoli
Nuclei
anti-Polymerase II
anti-PARP1
anti-UBF
D
+1
+550
+8200
28S
IgG anti-PARP1 or anti-UBF
HEK293T PARP1
HEK293T UBF
NIH3T3 PARP1
NIH3T3 UBF
Occupancy
+1 +550 +8200
E
meCpG unmethylated
HEK293T
NIH3T3
CpG methylated and unmethylated (%)
rDNA Total PARP1 UBF

Figure 1

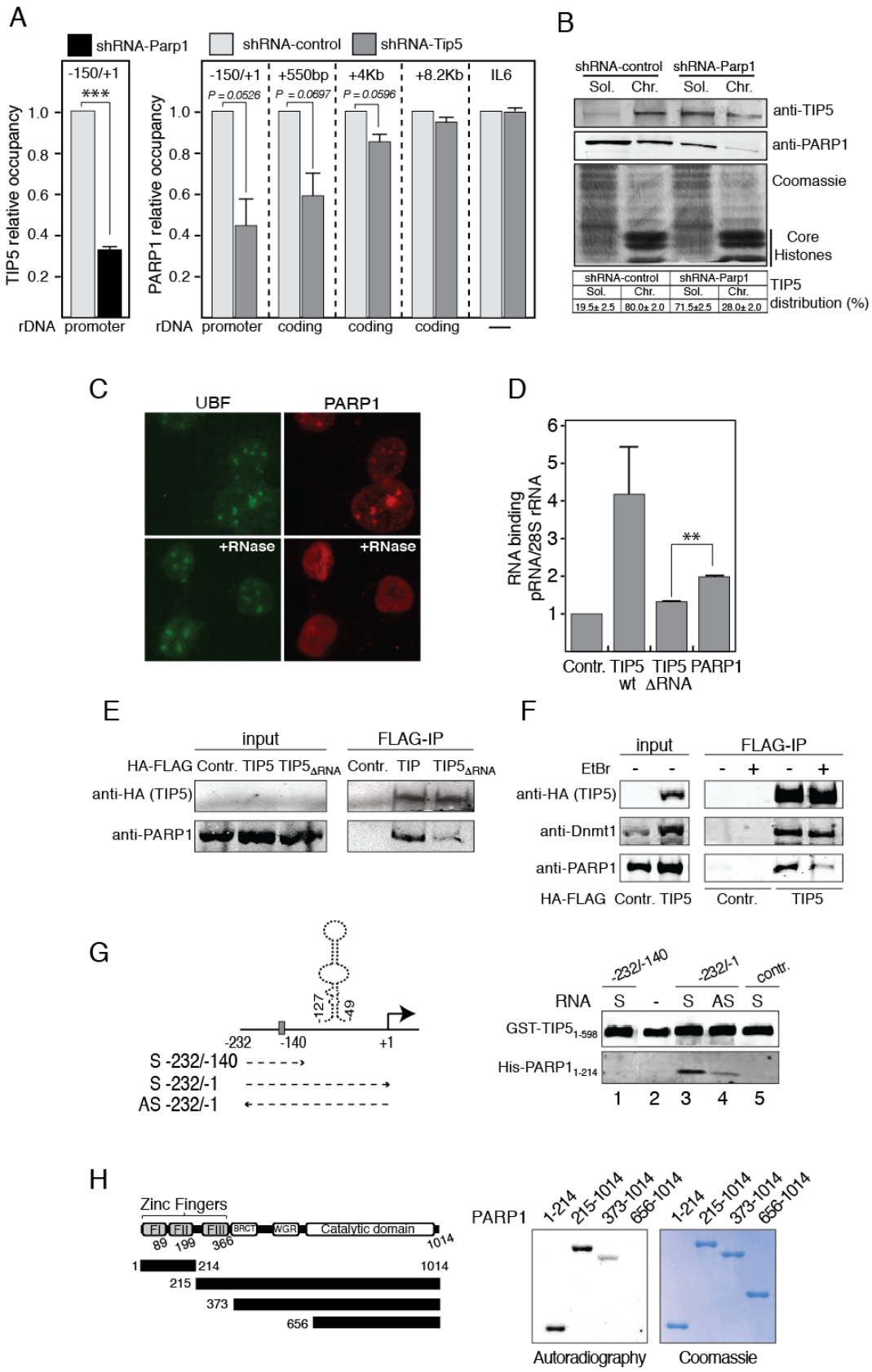

A
shRNA-Parp1
shRNA-control
shRNA-Tip5
-150/+1
***
TIP5 relative occupancy
1.0
0.8
0.6
0.4
0.2
rDNA promoter
PARP1 relative occupancy
-150/+1
+550bp
+4Kb
+8.2Kb
IL6
P = 0.0526
P = 0.0697
P = 0.0596
rDNA promoter coding coding coding
B
shRNA-control
shRNA-Parp1
Sol. Chr. Sol. Chr.
anti-TIP5
anti-PARP1
Coomassie
Core Histones
shRNA-control
shRNA-Parp1
Sol. Chr. Sol. Chr.
19.5± 2.5 80.0± 2.0 71.5±2.5 28.0± 2.0
TIP5 distribution (%)
C
UBF
PARP1
+RNase
+RNase
D
RNA binding pRNA/28S rRNA
6
5
4
3
2
1
**
Contr. TIP5 wt
TIP5 ΔRNA
PARP1
E
input
FLAG-IP
HA-FLAG Contr. TIP5 TIP5ΔRNA Contr. TIP TIP5ΔRNA
anti-HA (TIP5)
anti-PARP1
F
input
FLAG-IP
EtBr - - - + - +
anti-HA (TIP5)
anti-Dnmt1
anti-PARP1
HA-FLAG Contr. TIP5
Contr.
TIP5
G
-127
-49
-232
-140
+1
S -232/-140
S -232/-1
AS -232/-1
-232/-140
-232/-1
contr.
RNA S - S AS S
GST-TIP5 1-598
His-PARP1 1-214
1 2 3 4 5
H
Zinc Fingers
FI FII FIII BRCT WGR Catalytic domain
89 199 366 1014
1 214 1014
215
373
656
PARP1
1-214
215-1014
373-1014
656-1014
1-214
215-1014
373-1014
656-1014
Autoradiography
Coomassie

Figure 2

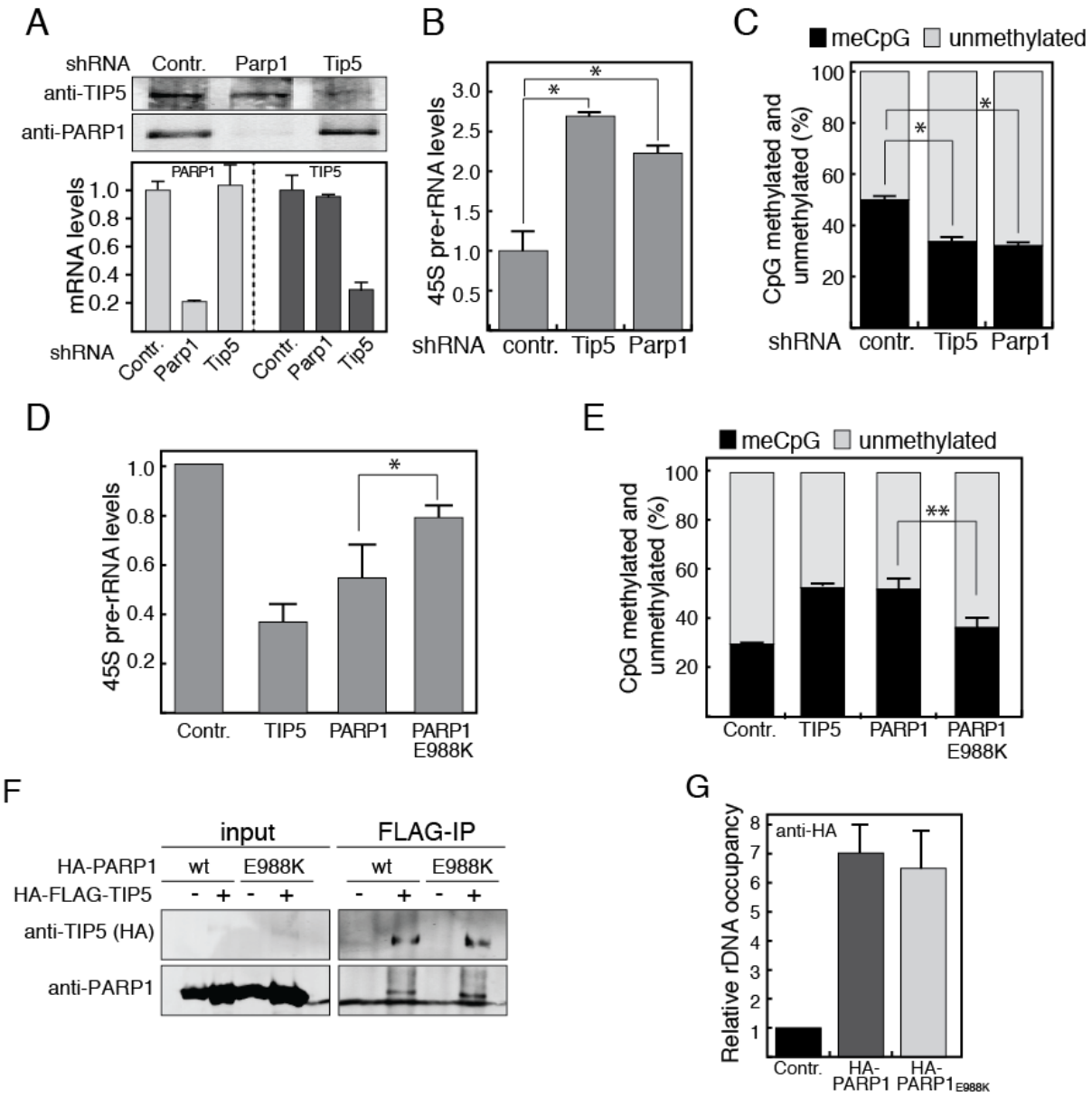

A
shRNA Contr. Parp1 Tip5
anti-TIP5
anti-PARP1
PARP1
TIP5
mRNA levels
shRNA Contr. Parp1 Tip5 Contr. Parp1 Tip5
B
45S pre-rRNA levels
shRNA contr. Tip5 Parp1
C
meCpG unmethylated
CpG methylated and unmethylated (%)
shRNA contr. Tip5 Parp1
D
45S pre-rRNA levels
Contr. TIP5 PARP1 PARP1 E988K
E
meCpG unmethylated
CpG methylated and unmethylated (%)
Contr. TIP5 PARP1 PARP1 E988K
F
input FLAG-IP
HA-PARP1 wt E988K wt E988K
HA-FLAG-TIP5 - + - + - + - +
anti-TIP5 (HA)
anti-PARP1
G
anti-HA
Relative rDNA occupancy
Contr. HA-PARP1 HA-PARP1E988K

Figure 3

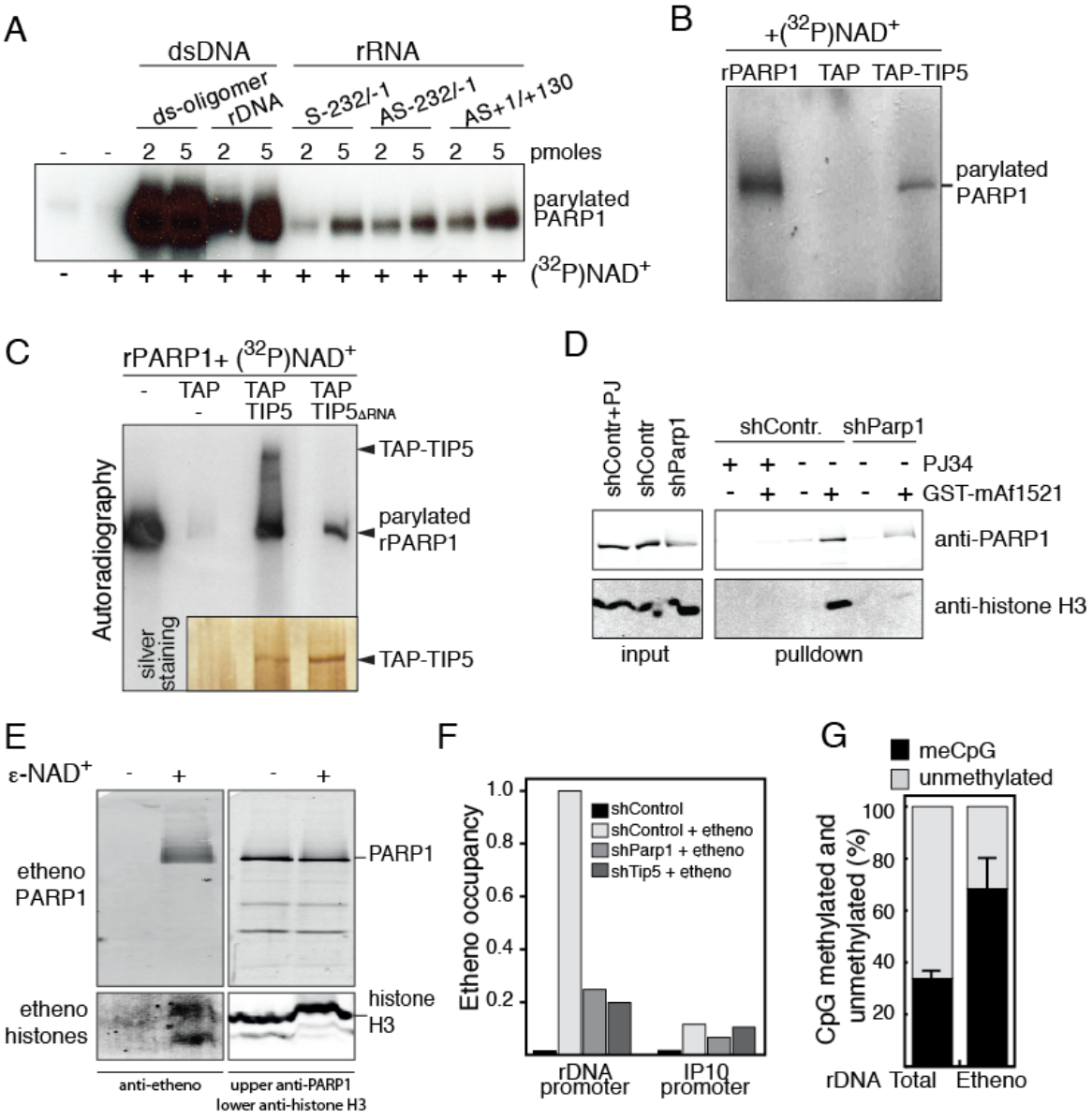

A
dsDNA
rRNA
ds-oligomer
rDNA
S-232/-1
AS-232/-1
AS+1/+130
- - 2 5 2 5 2 5 2 5 2 5 pmoles
parylated PARP1
- + + + + + + + + + + + (32P)NAD+
B
+(32P)NAD+
rPARP1 TAP TAP-TIP5
parylated PARP1
C
rPARP1+ (32P)NAD+
- TAP TAP TIP5 TAP TIP5ΔRNA
Autoradiography
TAP-TIP5
parylated rPARP1
silver staining
TAP-TIP5
D
shContr+PJ
shContr
shParp1
shContr.
shParp1
+ + - - - - PJ34
- + - + - + GST-mAf1521
anti-PARP1
anti-histone H3
input
pulldown
E
ε-NAD+ - + - +
PARP1
etheno PARP1
histone H3
etheno histones
anti-etheno
upper anti-PARP1
lower anti-histone H3
F
Etheno occupancy
1.0
0.8
0.6
0.4
0.2
shControl
shControl + etheno
shParp1 + etheno
shTip5 + etheno
rDNA promoter
IP10 promoter
G
meCpG
unmethylated
CpG methylated and unmethylated (%)
100
80
60
40
20
rDNA Total
Etheno

Figure 4

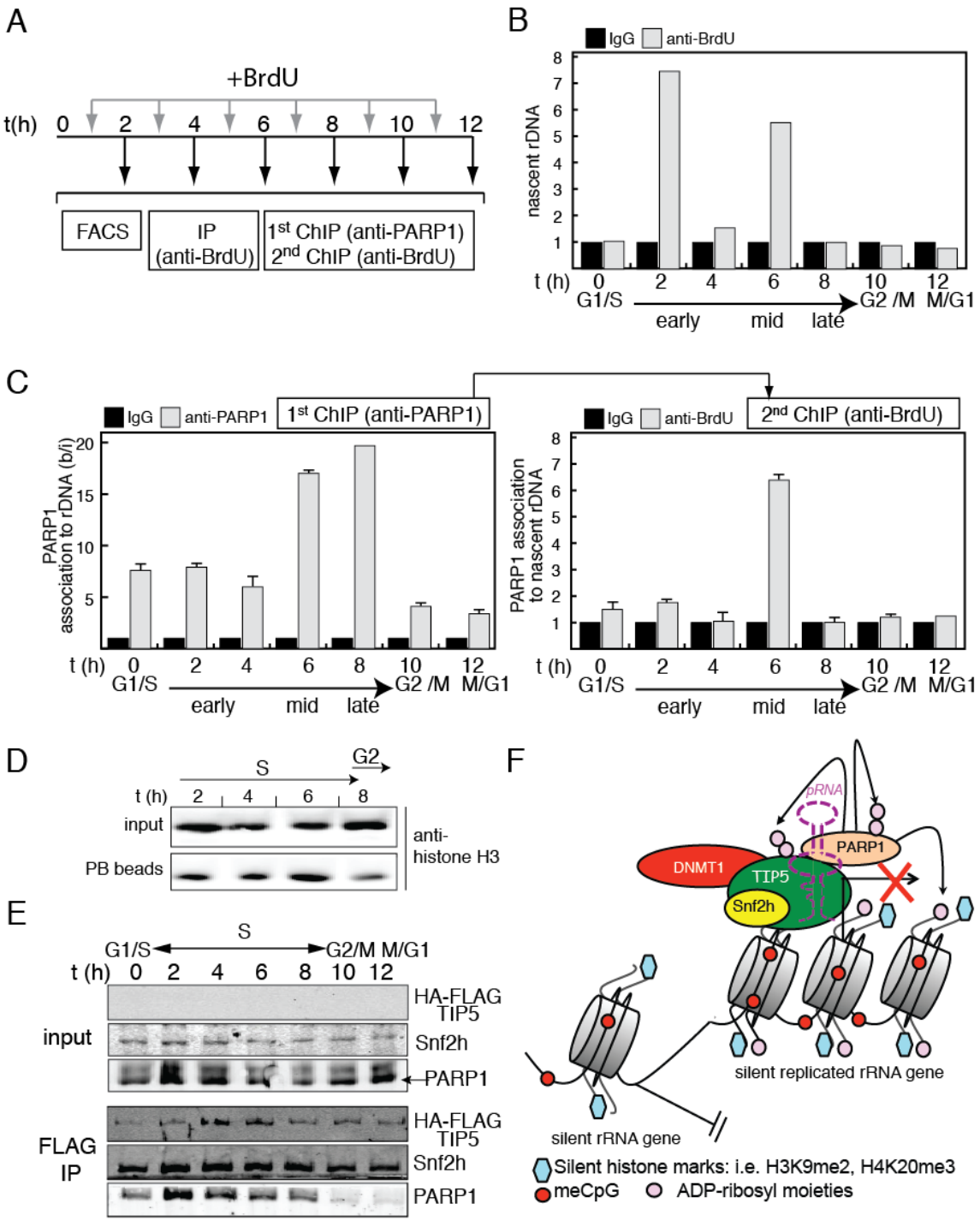
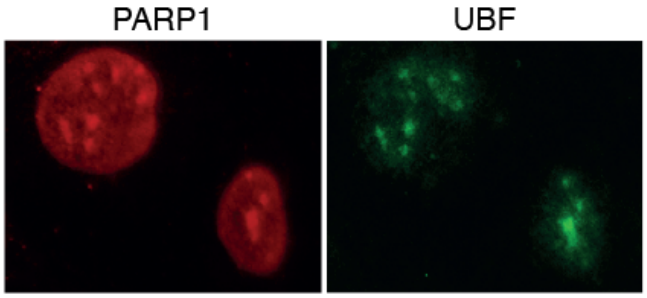

A
+BrdU
t(h) 0 2 4 6 8 10 12
FACS
IP (anti-BrdU)
1st ChIP (anti-PARP1)
2nd ChIP (anti-BrdU)
B
IgG anti-BrdU
nascent rDNA
t (h) 0 2 4 6 8 10 12
G1/S
G2 /M M/G1
early mid late
C
IgG anti-PARP1
1st ChIP (anti-PARP1)
PARP1 association to rDNA (b/i)
IgG anti-BrdU
2nd ChIP (anti-BrdU)
PARP1 association to nascent rDNA
D
S
G2
t (h) 2 4 6 8
input
PB beads
anti-histone H3
E
S
G1/S
G2/M M/G1
t (h) 0 2 4 6 8 10 12
input
FLAG IP
HA-FLAG TIP5
Snf2h
PARP1
F
pRNA
PARP1
DNMT1
TIP5
Snf2h
silent replicated rRNA gene
silent rRNA gene
Silent histone marks: i.e. H3K9me2, H4K20me3
meCpG
ADP-ribosyl moieties

Figure 5

A


PARP1
UBF

B

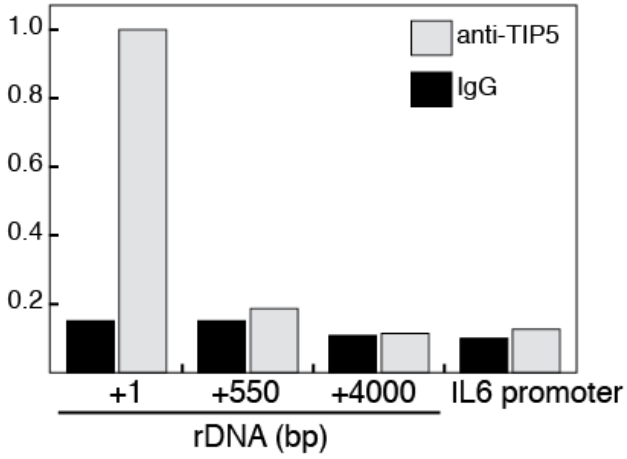

1.0
0.8
0.6
0.4
0.2
anti-TIP5
IgG
+1
+550
+4000
IL6 promoter
rDNA (bp)

Figure S1

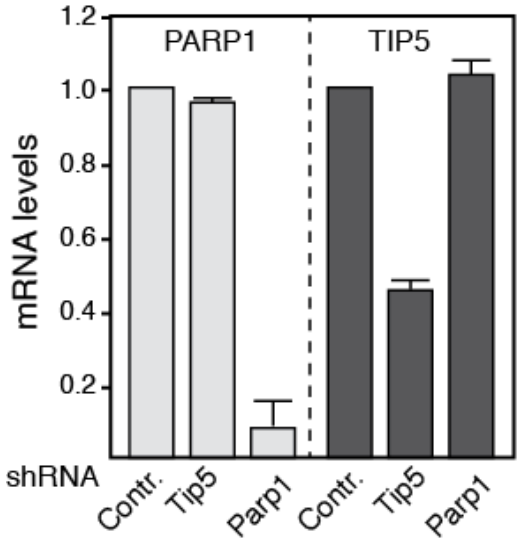

1.2
1.0
0.8
0.6
0.4
0.2
mRNA levels
PARP1
TIP5
shRNA
Contr.
Tip5
Parp1
Contr.
Tip5
Parp1

Figure S2

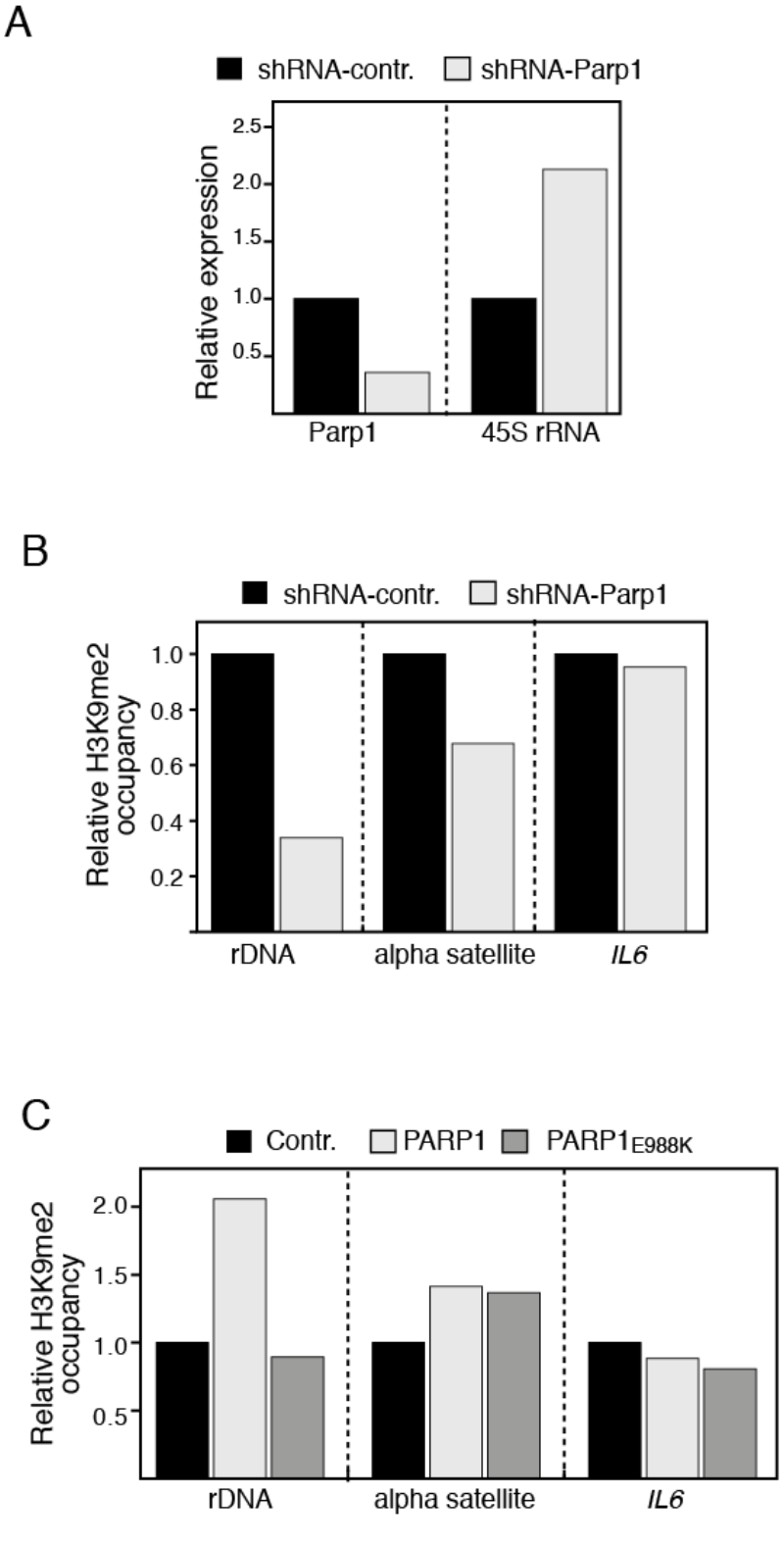

A
shRNA-contr.
shRNA-Parp1
Relative expression
2.5
2.0
1.5
1.0
0.5
Parp1
45S rRNA
B
shRNA-contr.
shRNA-Parp1
Relative H3K9me2 occupancy
1.0
0.8
0.6
0.4
0.2
rDNA
alpha satellite
IL6
C
Contr.
PARP1
PARP1E988K
Relative H3K9me2 occupancy
2.0
1.5
1.0
0.5
rDNA
alpha satellite
IL6

Figure S3

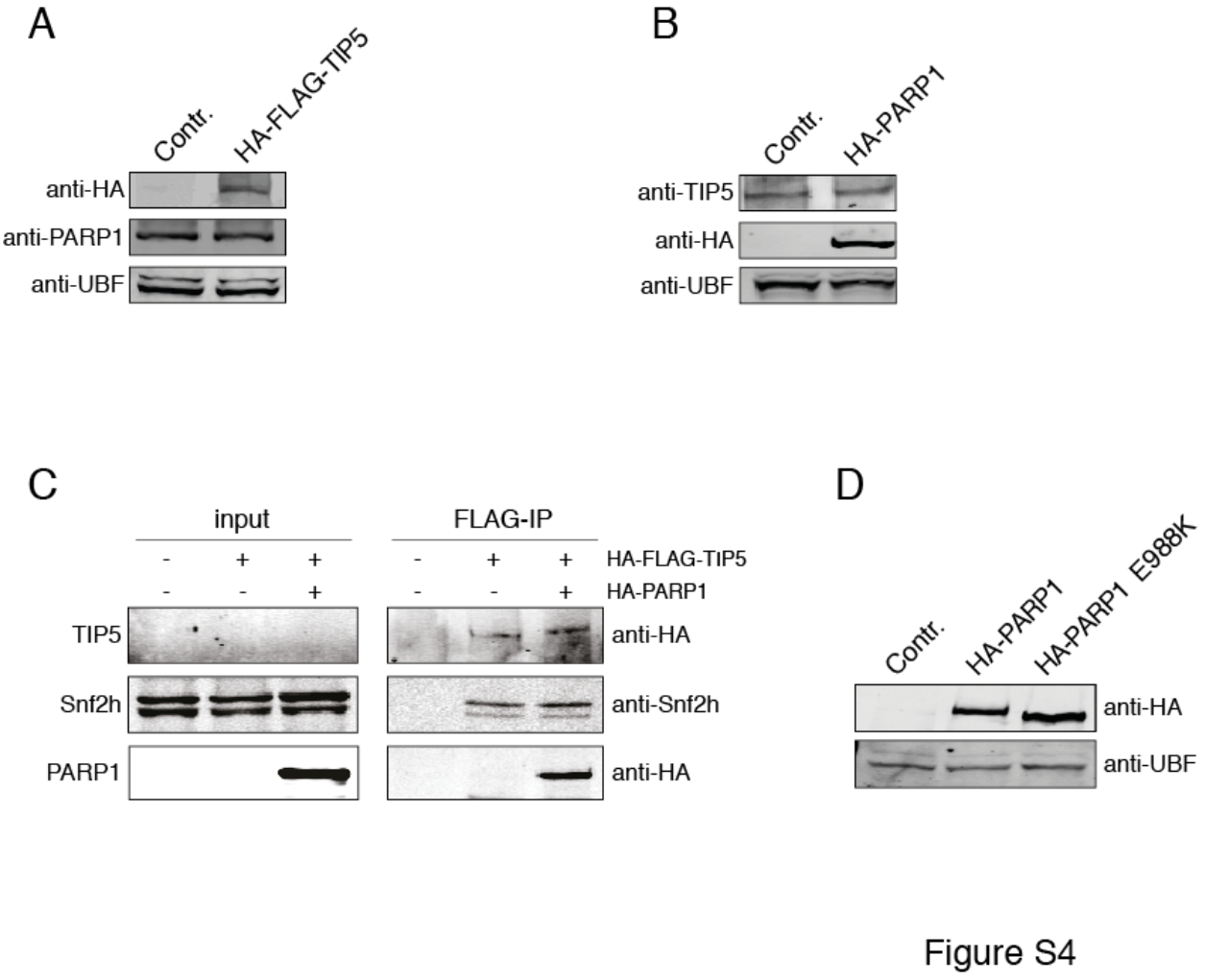

A
Contr.
HA-FLAG-TIP5
anti-HA
anti-PARP1
anti-UBF
B
Contr.
HA-PARP1
anti-TIP5
anti-HA
anti-UBF
C
input
FLAG-IP
- + + - + + HA-FLAG-TIP5
- - + - - + HA-PARP1
TIP5
Snf2h
PARP1
anti-HA
anti-Snf2h
anti-HA
D
Contr.
HA-PARP1
HA-PARP1 E988K
anti-HA
anti-UBF

Figure S4

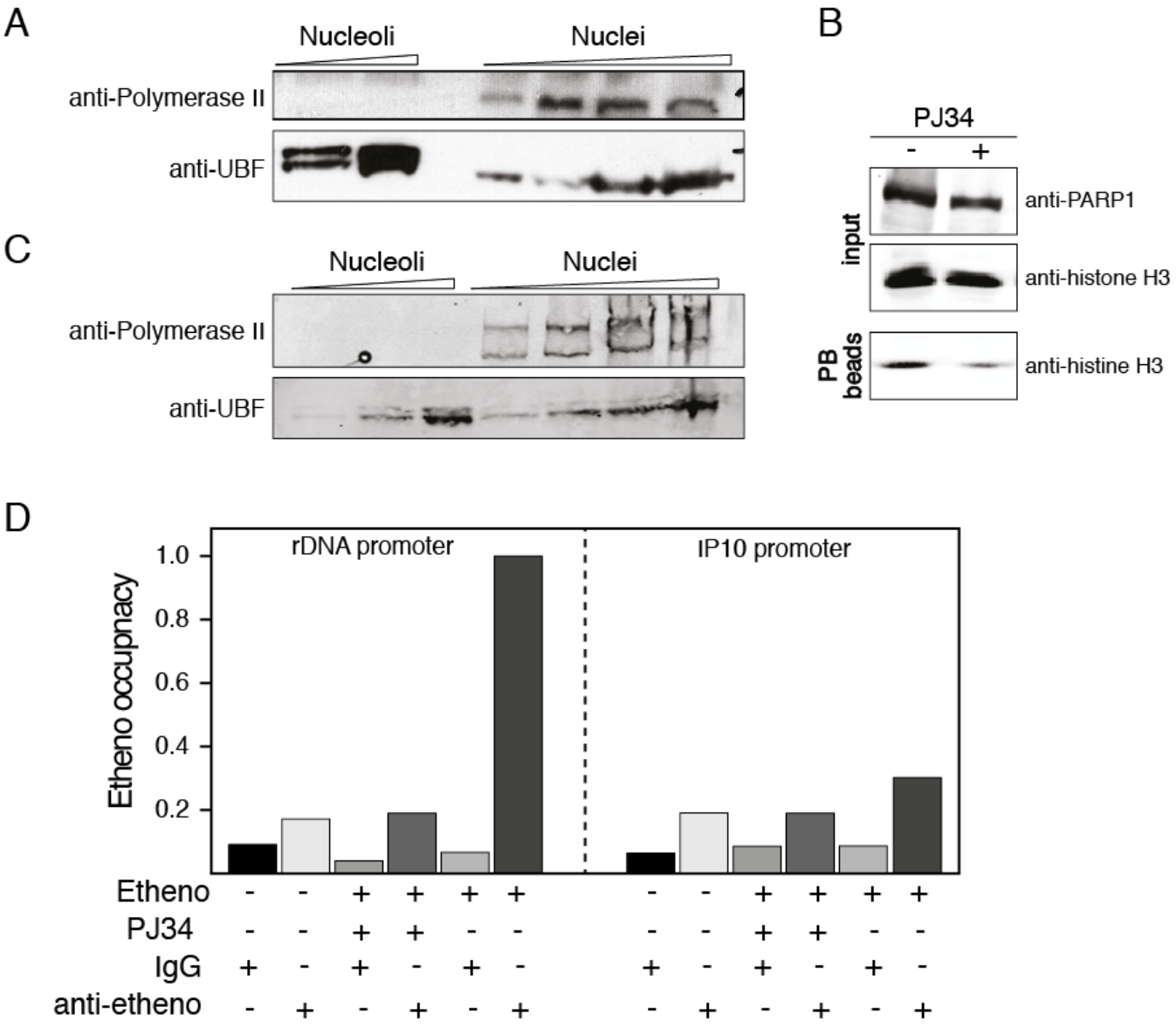

A
Nucleoli
Nuclei
anti-Polymerase II
anti-UBF
B
PJ34
-
+
input
anti-PARP1
anti-histone H3
PB beads
anti-histine H3
C
Nucleoli
Nuclei
anti-Polymerase II
anti-UBF
D
rDNA promoter
IP10 promoter
Etheno occupnacy
1.0
0.8
0.6
0.4
0.2
Etheno
PJ34
IgG
anti-etheno

Figure S5

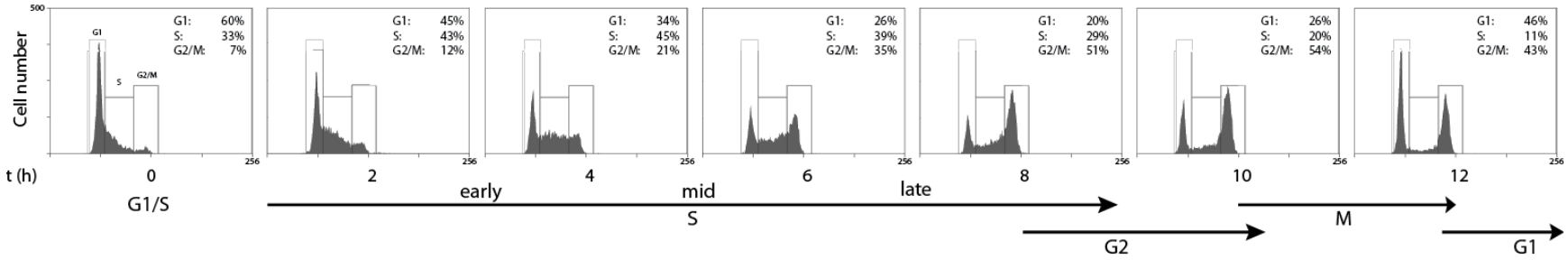

Cell number
500
G1
S
G2/M
G1: 60%
S: 33%
G2/M: 7%
G1: 45%
S: 43%
G2/M: 12%
G1: 34%
S: 45%
G2/M: 21%
G1: 26%
S: 39%
G2/M: 35%
G1: 20%
S: 29%
G2/M: 51%
G1: 26%
S: 20%
G2/M: 54%
G1: 46%
S: 11%
G2/M: 43%
256
t (h)
0
2
4
6
8
10
12
G1/S
early
mid
late
S
G2
M
G1

Figure S6

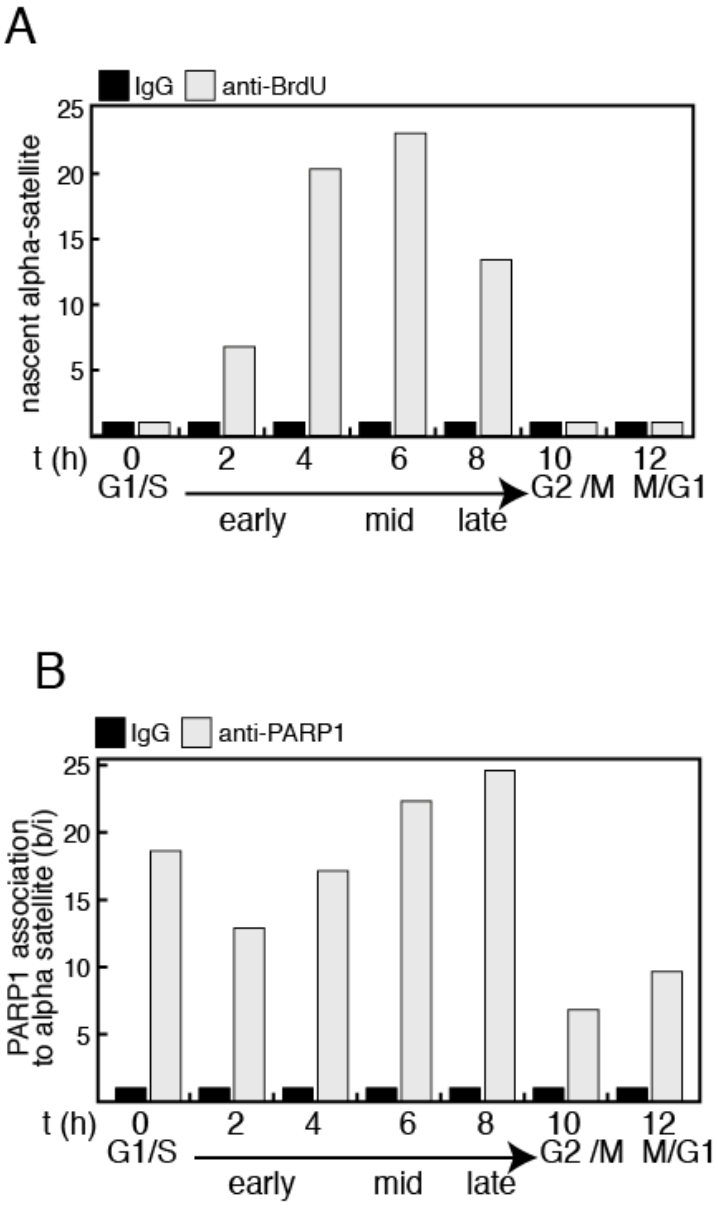

A
IgG
anti-BrdU
nascent alpha-satellite
25
20
15
10
5
t (h)
0
2
4
6
8
10
12
G1/S
G2 /M
M/G1
early
mid
late
B
IgG
anti-PARP1
PARP1 association to alpha satellite (b/i)
25
20
15
10
5
t (h)
0
2
4
6
8
10
12
G1/S
G2 /M
M/G1
early
mid
late

Figure S7

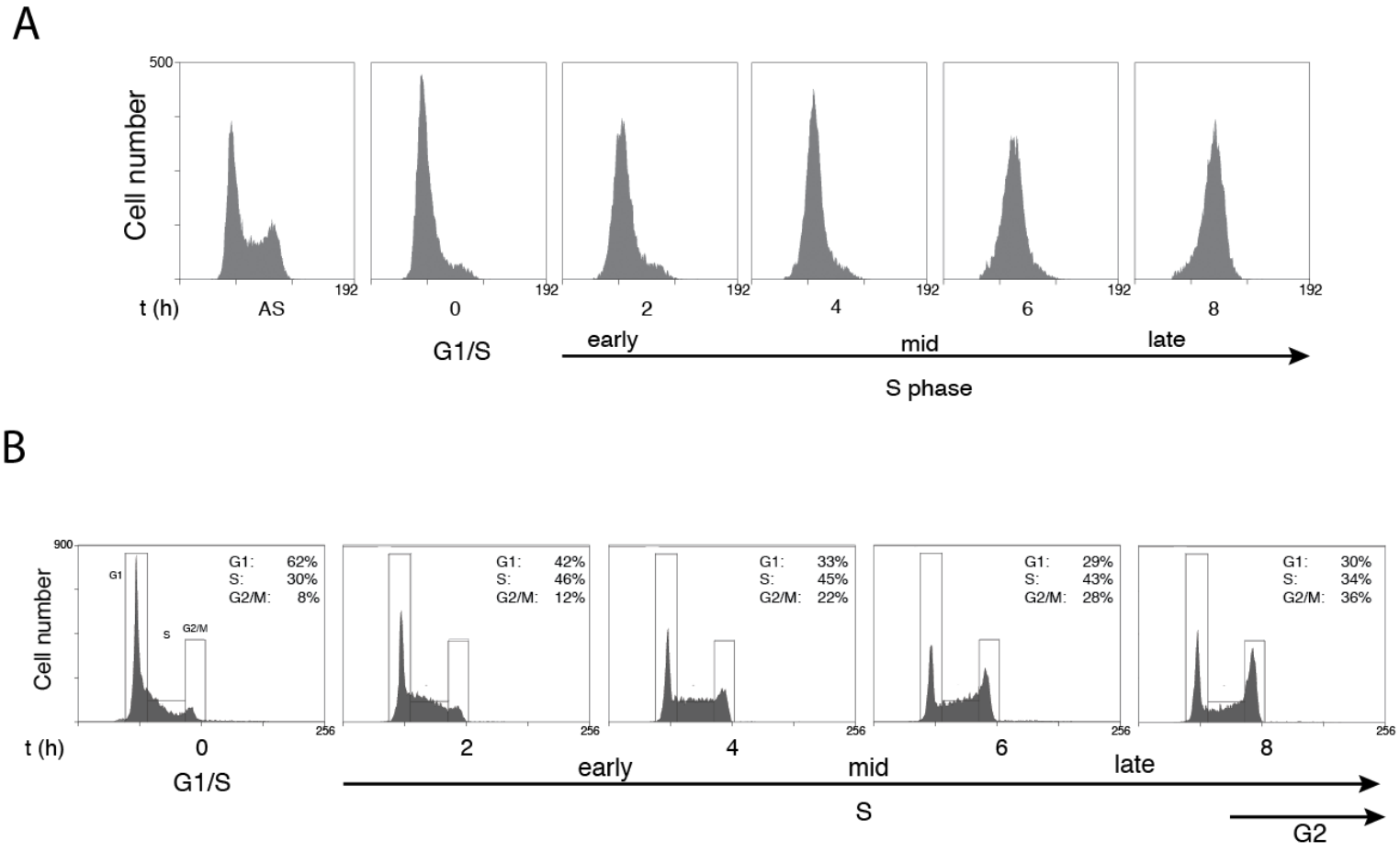

A
500
Cell number
192
t (h)
AS
0
2
4
6
8
G1/S
early
mid
late
S phase
B
900
Cell number
G1
S
G2/M
G1: 62%
S: 30%
G2/M: 8%
G1: 42%
S: 46%
G2/M: 12%
G1: 33%
S: 45%
G2/M: 22%
G1: 29%
S: 43%
G2/M: 28%
G1: 30%
S: 34%
G2/M: 36%
256
t (h)
0
2
4
6
8
G1/S
early
mid
late
S
G2

Figure S8

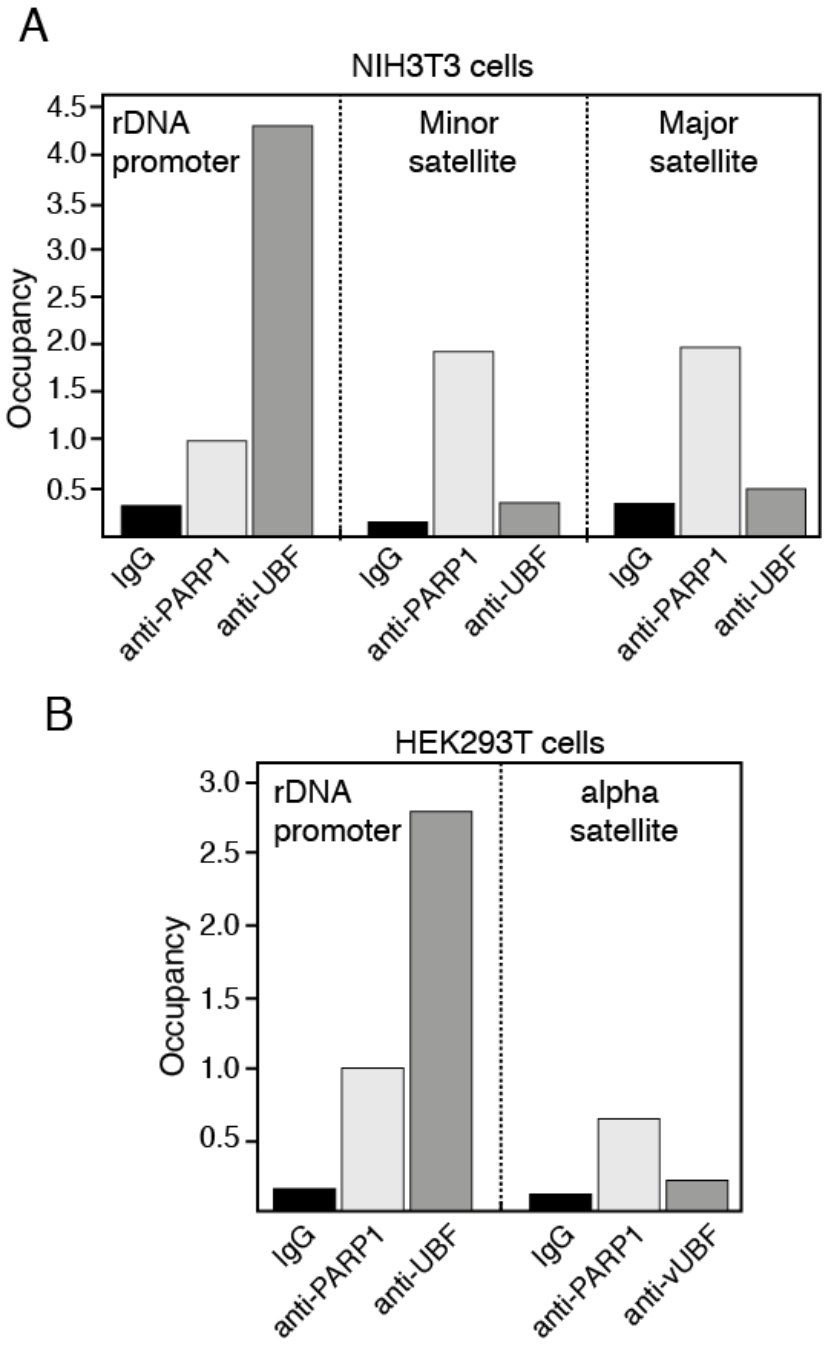

A
NIH3T3 cells
rDNA promoter
Minor satellite
Major satellite
Occupancy
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
IgG
anti-PARP1
anti-UBF
IgG
anti-PARP1
anti-UBF
IgG
anti-PARP1
anti-UBF
B
HEK293T cells
rDNA promoter
alpha satellite
Occupancy
3.0
2.5
2.0
1.5
1.0
0.5
IgG
anti-PARP1
anti-UBF
IgG
anti-PARP1
anti-vUBF

Figure S9

4 Discussion

In this work, data are presented that describe 1) the function of TIP5, the subunit of the NoRC complex, and of rDNA silencing in cell metabolism and in nuclear/nucleolar architecture (see **3.1.1**); 2) the role of PARP1 in the establishment and inheritance of silent rDNA chromatin (see **3.1.2**).

4.1 The NoRC complex mediates the heterochromatin formation and stability of silent rRNA genes and centromeric repeats

The aim of this work was to elucidate the role of TIP5 and rDNA silencing in cell metabolism and in nuclear/nucleolar architecture. The experimental strategy here applied was the shRNA-mediated knockdown of TIP5, the NoRC subunit recruiting repressor complexes at the rDNA locus. The data of this work unravelled that depletion of TIP5 in NIH3T3 cells impaired rDNA silencing, upregulated rRNA transcription levels, altered nucleolus structure, accelerated cell proliferation rates and induced cell transformation. Moreover, the results of this work demonstrated that TIP5 not only mediated the establishment of rDNA silencing but also the formation of perinucleolar heterochromatin that contains centric and pericentric repeats. The data also determined that TIP5-mediated heterochromatin formation is indispensable for stability of silent rRNA genes and of major and minor satellite repeats. Taken together, these findings point to a role of TIP5 in protecting genome stability and suggest that it can play a role in the cellular transformation process. The content of this work was recently published in *EMBO J.*

4.1.1 Depletion of TIP5 impairs rDNA silencing and upregulates rDNA transcription

Here it is demonstrated that after depletion of TIP5 the amounts of silent genes decrease, underscoring the role of TIP5 in establishing silent rDNA chromatin. Notably, although TIP5 binds specifically to the rDNA promoter, the levels of rDNA CpG methylation levels after TIP5 depletion were reduced over the entire rRNA gene, underscoring the role of TIP5 in initiating local silencing events, which then spread over

the whole rDNA unit. Consistent with the loss of silent epigenetic marks (CpG methylation and silent histone marks), cells depleted of TIP5 displayed increased rRNA transcription levels. These results are also supported by recent data that showed that depletion of TIP5 in HEK293T and CHO-K1, two mammalian high-producer cell lines, decreased rDNA silencing, upregulated rRNA transcription, enhanced ribosome synthesis and increased production of recombinant proteins, linking the levels of rDNA silencing with ribosome biogenesis [131]. Surprisingly, the data unraveled that the enhancement of rRNA transcription in TIP5-depleted cells did not depend on the number of active genes. An analysis performed by tracking the rRNA genes using a polymorphism at +42/43 showed that the absolute number of active genes remained unchanged after depletion of TIP5. In contrast, the absolute number of silent methylated rRNA genes decreased after knockdown of TIP5 (see **4.1.4**). As the increase of rRNA synthesis after TIP5 depletion does not correlate with the number of active genes, the results suggest that lack of TIP5 enhance transcription rates of rRNA active genes. These results strengthen the view that rDNA transcription is preferentially modulated by altering the transcriptional activity of each gene and not by altering the number of genes [102]. It seems, therefore, that TIP5 and/or presence of heterochromatic silent rDNA repeats might indirectly affect the transcription rate of active genes, probably by enriching the nucleolar compartment of the chromatin repressor complexes. However, we cannot exclude the possibility that upregulation of rDNA transcription is a consequence of genome instability that caused the acquisition of aberrant mechanisms of rDNA transcriptional regulation, thus representing an advantage for the elevated protein synthesis necessary for high proliferative rates.

4.1.2 Depletion of TIP5 induces cellular transformation

These results implicated that TIP5-depleted cells possessed higher proliferation rates, and proliferated beyond confluence, forming cellular foci and peeling off the culture surface in large mass, a typical characteristic of transformed cells. These data suggest that depletion of TIP5 and impairment of rDNA silencing can contribute to cellular transformation and strengthen the intimate link between rDNA transcription, cell

growth/proliferation and cancer. In cancer cells, rDNA transcription is enhanced, contributing to increased production of ribosomes and protein synthesis of the rapidly proliferating tumors [70, 108]. Disruption in one or more of the steps that control protein biosynthesis has been associated with alterations in the cell cycle and regulation of cell growth [70]. Consistent with this, recent data implicated that depletion of TIP5 and impairment of rDNA silencing enhances ribosome synthesis and increases protein production [131]. A lower content of rDNA methylation was reported for several tumors [109-111], strengthening the notion of the role of CpG methylation in repressing rDNA transcription [103]. Moreover, rDNA CpG methylation levels were found to be higher in ovarian cancer patients with long progression survival as compared with that in patients with short survival, an indication that rDNA silencing levels may influence cell growth properties essential for active tumor proliferation and tumor aggressiveness [107]. Future studies will address whether TIP5 levels correlate with the severity of tumors (malignant and metastatic).

4.1.3 TIP5 mediates heterochromatin formation at centric and pericentric repeats

In this work (see **3.1.1**), it is further shown that depletion of TIP5 and reduction of rDNA silencing levels affects the nucleolar structure and impairs the formation of condensed chromatin within and in close proximity of the nucleolus. Alterations in the nucleolar structure were often detected by changes in the levels of rRNA synthesis [132]. As consequence of elevated nucleolar activities, cancer cells show enlarged nucleoli, which are commonly used by pathologists to identify tumor formation [70]. Consistent with this, nucleoli in TIP5 depleted cells not only diminished in number but also showed enlarged structure, a characteristic indication of elevated rDNA transcription activities. Structural alterations were also detected at the centromeric loci in TIP5 depleted cells by immunostaining with antibodies against the core kinetochore CENP-A. After depletion of TIP5, CENP-A stained foci decreased in number and increased in size, linking TIP5 and/or rDNA silencing and formation of centromeric heterochromatin. Heterochromatin of pericentric and centric regions was previously described to localize also alongside the nucleolus [133, 134]. This close proximity to the nucleolus is mainly explained by the

fact that the rRNA genes are positioned very close to the centromeres [135, 136]. Due to the linear proximity, centromeres of chromosomes bearing rDNA repeats associate with nucleoli. Notably, also chromosomes devoid of rRNA genes have their centromeres associated with the nucleolus at a frequency more than that expected for a random distribution [133]. The basis of this association probably relies on the linear proximity along the chromosome and on the repeated nature of DNA sequence, which provides multiple binding sites for specific proteins capable of forming multimeric complexes. Several pieces of evidences indicate that silent rDNA arrays are located in the extranucleolar space, frequently associated with the perinucleolar heterochromatin [137]. CpG-methylated rRNA genes ("stable" silent copies) were shown to assemble adjacent to the perinucleolar heterochromatin composed of centric repeats [138]. On the other hand, active rDNA repeats are located inside the nucleolus within the dense fibrillar components. Our results showed that depletion of TIP5 not only reduced the levels of silent histone marks and CpG methylation at the rDNA locus but it also decreased the levels of two typical silent histone modifications (H3K9me3 and H4K20me3) at centric and pericentric repeats. Although our ChIP data indicate a weak and transient interaction, association of TIP5 with the centromeric protein CENP-A suggests that this interaction indeed takes place. As CpG-methylated rRNA genes were previously shown to assemble adjacent to the perinucleolar heterochromatin [138], such spatial and linear closeness may allow TIP5, bound to silent rRNA genes, to interact with centric repeats and to aid in establishing heterochromatic structures using similar mechanisms as used to silence the rDNA locus **(Figure 7)** [104, 114]. Alternatively, the repressive chromatin of silent rDNA copies may affect the centric and pericentric heterochromatin either by spreading mechanisms or by creating a nucleolar/perinucleolar compartment enriched in chromatin repressor complexes. In both cases, decrease of rDNA silencing after TIP5 depletion would affect the spreading of heterochromatin and reduce the levels of repressor complexes within and nearby the nucleolus. Notably, a role of the perinucleolar compartment in mediating the incorporation of repressive chromatin factors was recently discussed for the establishment of the inactive X-chromosome (Xi). During mid-to-late S-phase, 80 - 90 % of the inactive X-chromosome contact the nucleolus and reside within an Snf2h-enriched ring. Furthermore, the data showed that autosomes carrying ectopic X-

inactivation center sequences are also targeted to the perinucleolar compartment. Deletion of the X-inactive specific transcript (*Xist*) results in a loss of nucleolar association and inability to maintain Xi chromatin, leading to Xi reactivation at single gene level. These data proposed that Xi must continuously visit the perinucleolar compartment to maintain its epigenetic state [139]. Interestingly, TIP5 and rDNA silencing seems also to play a role during maintenance of inactive X chromosome structure (Santoro, unpublished data).

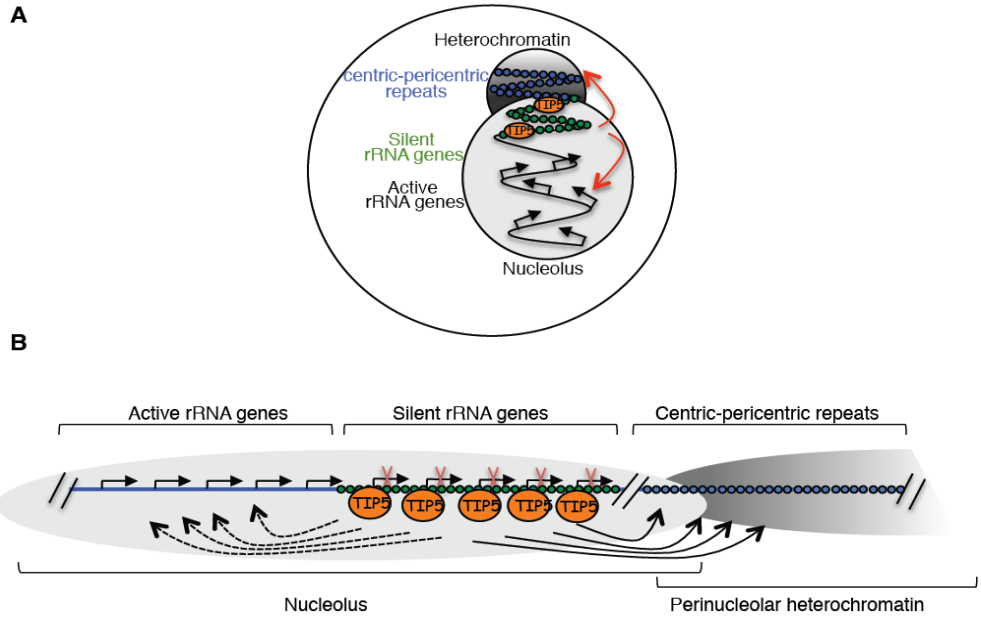


Figure 7 | TIP5 mediates the heterochromatin at the nucleolar/perinucleolar associated chromatin A model showing the role of TIP5 in establishing heterochromatin at regions located adjacent to the nucleolus. The cellular (A) and linear (B) distribution of active/silent rRNA genes and centromeric heterochromatin within the nucleolus and at the perinuclear periphery. In this model, it is also proposed that TIP5 and silent rRNA copies have a role in mediating the transcriptional activity of active rRNA genes (modified from [140]).

The results of this work suggest that the role of silent rRNA genes and TIP5 go beyond regulation of rRNA synthesis and that they can play an important role at the level of nuclear/nucleolus chromatin architecture. Indeed, the presence of silent rDNA copies was also detected in a yeast strain containing about 42 rDNA copies that, until now, were considered *bona fide* all competent for transcription [102, 141]. Although the rRNA genes of this strain are highly transcribing to compensate for the absence of about 100 copies, a fraction of 10-20% of rRNA genes persists to remain inaccessible to psoralen

(nucleosomal silent rDNA fraction) [141]. The presence of silent rRNA copies in a strain where all the rRNA genes should be dedicated to transcription suggests that the presence of silent copies is indispensable and that probably their role is tightly linked to chromosomal structural organization.

Taken together, these results demonstrated that TIP5 is not only involved in formation of rDNA silencing but it also affects heterochromatin structures of centric and pericentric repeats.

4.1.4 TIP5 protects the stability of silent rDNA, centric and pericentric repeats

This work showed that depletion of TIP5 impairs genome stability, which is known to be a principal molecular step in cancer formation. Formation of specific heterochromatic domains is crucial for genome stability [142, 143] and is generally thought to serve as a mechanism ensuring repeat stability by limiting access to the recombination machinery. A large body of evidences indicates that maintenance of silent rDNA chromatin plays an important role in the stability of rRNA repeats. In the yeast *S. cerevisiae*, recruitment of the nucleolar protein complexes RENT (regulator of nucleolar silencing and telophase exit) and Cohibin to rDNA suppresses unequal recombination at the rDNA repeats [144], linked to the ability of these complexes to induce rDNA silencing. This suppression is seemingly linked to the ability of these complexes to induce rDNA silencing. Similarly, segments of rRNA genes and satellite repeat arrays become dispersed in *Drosophila* mutants that are defective in the histone methyltransferase Su(var)3-9, in HP1 also known as Su(var)2-5); or in several genes involved in the RNA interference (RNAi) pathway [145]. Consistent with this, knockdown of TIP5 in NIH3T3 cells not only impairs formation of heterochromatin at rRNA genes and satellite repeats, but it also induces specific loss of silent rDNA repeats and of major and minor satellites replicating in the mid-late S-phase. Until now, the repetitive nature of the rRNA genes represented a limit in determining which rRNA genes (active or silent) undergo instability in the absence of these chromatin repressor complexes. By tracking rRNA genes with polymorphic variations, it was shown that TIP5-mediated heterochromatin formation

specifically protects CpG methylated (stable) silent rRNA genes from illicit recombination events whereas active genes are not affected. As formation and maintenance of heterochromatic structures is crucial for genome stability, the data suggest that TIP5-mediated heterochromatin plays an important role in protecting the genome from inappropriate chromosomal rearrangements and that the structure of "stable" silent rRNA genes is important for nucleolus/nuclear chromatin architecture.

Although it remains to be estimated to which extent the genome instability or enhancement of rDNA transcription in TIP5-depleted cells contributed to the transformed phenotype, our results provide evidences that the TIP5-mediated heterochromatin has a crucial role in protecting genome stability and regulating rDNA transcription, thus contributing to the cellular transformation process.

4.2 PARP1 is recruited to the rRNA genes *via* non-coding RNA and mediates inheritance of silent rDNA chromatin

The aim of this work was to determine the mechanistic insights of how silent rDNA chromatin structure is propagate during cell division. This work led to the identification of the poly(ADP-ribose) polymerase-1 (PARP1) as a critical component of the NoRC complex that establishes and maintains silent rDNA chromatin. The data showed that: 1) PARP1 associates with TIP5 and that this interaction is mediated by pRNA; 2) PARP1 is associated with the silent rRNA genes after the passage of the replication fork; silent rDNA chromatin is a specific substrate for ADP-ribosylation and that the enzymatic activity of PARP1 is necessary to establish rDNA silencing. , implying a role of PARP1 in the inheritance of rDNA silent chromatin during cell division. Taken together the data unravelled a novel function of PARP1 and ADP-ribosylation that serves to inherit silent rDNA chromatin structures during cell division. In addition, the results support a model of how ncRNAs serve as scaffolds by providing binding surfaces to assemble selected chromatin modification enzymes, thereby specifying the pattern of epigenetic modifications on target genes. This work was recently submitted for publication.

4.2.1 PARP1 associates with TIP5 *via* pRNA

The non-coding pRNA sequences from nucleotides -127 to -49 in mouse were previously shown to form a conserved hairpin structure that is specifically recognized by the TIP5-TAM domain (see **1.5.3**). Upon pRNA binding, TIP5 undergoes a conformational change that was proposed to facilitate the interaction with other proteins required for rDNA silencing [123]. In this work, we identified PARP1 as a TIP5 interacting protein whose association is dependent on pRNA. The role of RNA in PARP1-TIP5 association is also supported by the impairment of PARP1 nucleolar localization after treatment with RNase, a treatment that was previously shown to displace TIP5 from the nucleolus [90]. Similarly to pRNA, the long intergenic non-coding RNA (lincRNA) HOTAIR was recently shown to act as scaffold by providing

binding surfaces to assemble the polycomb repressive complex 2 (PRC2) and the histone demethylase LSD1 at target genes. The ability to tether two distinct complexes enables RNA-mediated assembly of PRC2 and LSD1 and coordinates targeting of PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and H3K4 demethylation [146]. Thus, our work indicated that pRNA serves as scaffolds by providing binding surfaces to assemble selected chromatin modification enzymes.

4.2.2 PARP1 associates with silent rRNA genes after the passage of the replication forks

Maintenance and transmission of proper chromatin organization is fundamental for genome stability and function in eukaryotes. During DNA replication, both heterochromatin and euchromatin are disrupted ahead of the replication fork and are then reassembled into their original epigenetic states behind the fork. How chromatin domains are restored on new DNA and transmitted through mitotic cell division remains a fundamental question in biology, with implications for development and complex diseases like cancer [147]. This work demonstrated that PARP1 associates with silent methylated rRNA genes after the passage of the replication fork, implying a role in the inheritance of silent rDNA chromatin. The timing of the binding of PARP1 to rDNA correlates well with the association of mature pRNA and TIP5 with rDNA [91, 115]. pRNA might guide TIP5 to rDNA *via* triple helix formation [148] or by stabilizing TIP5 association after recruitment mediated by the transcription terminator factor TTF-1 [105]. In both cases, the reported TIP5 conformational change induced upon binding of pRNA [123] might favour the association of PARP1 and the subsequent recruitment to newly synthesized rDNA. The role of pRNA in mediating the association of PARP1 with TIP5 and binding to newly synthesized silent rRNA copies strongly supports the idea that specific non-coding RNA can potentially direct complex patterns of chromatin states at specific genes in a spatially and temporally organized manner, which was also proposed for the maintenance of inactive X-chromosome chromatin [139]. Notably, after the completion of S phase TIP5-PARP1 interaction is impaired. These results suggest that this association is not only critical for a specific time window of cell cycle but is also

dynamic. Release of PARP1 from TIP5-pRNA might be modulated by posttranslational modifications. Recently, binding of Ezh2 to non-coding RNA HOTAIR and *Xist* has been reported to be upregulated when Ezh2 was phosphorylated by cyclin-dependent kinase 1 (CDK1) at threonine residues 345 and 487 in a cell cycle-dependent manner. A phosphor-mimic at residue 345 increased HOTAIR ncRNA binding to Ezh2, while the phosphor-mimic at residue 487 was ineffectual. An Ezh2 domain comprising T345 was found to be important for binding to HOTAIR and the 5' end of *Xist* [149]. Many studies suggested that PARP1 activity could be regulated by phosphorylation involving several kinases that are parts of important regulatory pathways [150]. Whether and how a cell cycle regulated phosphorylation of PARP1 might influence the binding stability with TIP5-pRNA will be addressed in our future studies.

4.2.3 PARP1 is a critical component of the machinery that maintains silent rDNA chromatin during cell division

This work showed that PARP1 has the ability to establish silent rDNA chromatin and to repress rRNA transcription. Similarly to TIP5 (this study), knockdown of PARP1 in mouse and human cells enhances rRNA synthesis, decreased the meCpG levels of rDNA and the association of histone H3K9me2, a histone mark associated with silent rDNA chromatin [104]. Consistent with these results, overexpression of PARP1 (and also of TIP5), decreased rRNA transcription, increased rDNA methylation and the association of H3K9me2. These data unravelled a novel function of PARP1 that is to establish silent rDNA chromatin. As binding of PARP1 to rDNA occurs after the replication of silent rRNA genes, these results suggest that PARP1 is implicated in the inheritance and propagation of silent rDNA chromatin during cell division.

Early studies determined a link between PARP1 and the DNA replication process. PARP1 was shown to co-localize with replication foci throughout S phase and to interact with several DNA replication proteins, many of which were poly ADP-ribosylated [151-154]. In addition, PARP activity was found to be enhanced in replicating cells [155], in the vicinity of replication forks [156] and in newly replicated chromatin [157]. Notably, the role of PARP1 in DNA replication was mainly described in combination with DNA

repair and recombination. For example, PARP1 was shown to collaborate with the repair protein Mre11 to promote replication fork restart after release from replication blocks. In line with this, PARP1 and PARP2 were described to be required for hydroxyurea-induced homologous recombination to promote cell survival after replication blocks [158, 159]. Whether PARP1 plays other roles during DNA replication was so far not yet addressed. The association of PARP1 with the NoRC/pRNA complex and its ability to silence rRNA genes strongly suggest a critical role in the inheritance of silent rDNA chromatin during cell division. In support of this, recent results identified PARP1 as SMARCAD1 interacting protein in several human cell lines [160]. SMARCAD1 is recruited to sites of DNA replication and ensures that silenced loci, such as pericentric heterochromatin, are correctly re-established. Although the role of PARP1 in the maintenance of pericentric heterochromatin mediated by SMARCAD1 was not explored by this study, we considered that this hypothesis could not be excluded. As discussed above (see **4.1**), TIP5 binds to major and minor satellite DNA and knockdown of TIP5 impairs heterochromatin formation at these repeats, leading to loss of mid-late replicating sequences [140]. Lack of PARP1 is associated with severe chromosomal instability, characterized by increased frequencies of chromosome fusions and aneuploidy [161]. Interestingly, we found that PARP1 binds to mouse centric repeats and human alpha satellite DNA and that depletion or overexpression of PARP1 affects the H3K9me2 levels at the alpha satellite DNA. If the function of PARP1 in the propagation of rDNA silent chromatin can be linked to the maintenance of centric- pericentric heterochromatin will be addressed in our future work.

4.2.4 The enzymatic activity of PARP1 is required for rDNA silencing

The data in this work indicate that nucleolar histones are parylated by PARP1 and that PARP1 can parylate itself and other components of the NoRC complex, including TIP5. The observed increase in nucleolar histone parylation, at the time when rRNA genes are replicated and bound by PARP1, strongly suggests a functional link between parylation and the re-establishment of silent rDNA chromatin. Consistent with this, $PARP1_{E988K}$ (a mutant lacking the ability to generate PAR polymers) was less efficient in repressing rRNA transcription and in establishing silent rDNA chromatin. Although the association with TIP5 and binding to silent rDNA was not compromised, rRNA genes bound by $PARP1_{E988K}$ were less methylated. The enzymatic activity of PARP1 was proposed as the switch event that might distinguish between a PARP1 with co-repressor and co-activator function. The ability to disrupt chromatin structure by parylating histones and destabilizing nucleosomes was one of the earliest functional effects of PARP1 to be characterized [52, 58-60, 162]. The role of parylation in decondensing chromatin finds its best example in the rapid accumulation of PAR at heat shock loci in response to heat shock in *Drosophila* [62]. dPARP is required for heat shock-induced ‘‘puffing’’ (i.e., chromatin decondensation) and knockdown of dPARP or treatment with a PARP inhibitor prevents heat shock-induced nucleosome loss and enhanced transcription at the Hsp70 gene [63]. However, examples exist where PARP1, when acting as co-activator, does not require its enzymatic activity [64-66]. Our data pointed out that the enzymatic activity of PARP1 is not only limited to processes where PARP1 acts as co-activator. We showed that PARP1-mediated parylation affects formation of rDNA silencing and that silent rDNA chromatin is a substrate for parylation. These results are consistent with previous studies showing that many of the *Drosophila* modified proteins detectable with anti-PAR antibodies were particularly enriched in nucleoli and in the heterochromatic chromocenter regions [57]. Taken together, the results suggest that the propagation of silent marks at the rDNA locus requires PARP1 activity. Notably, these results showed that RNA has the ability to activate PARP1. Thus, pRNA might not only mediate the association of PARP1 with TIP5 but also modulate the enzymatic activity of PARP1. There are many possibilities by which parylation can act to establish silent rDNA chromatin. PARP1 could covalently modify another protein to

activate the rDNA silencing process. The results showed in this work indicated that components of the NoRC complex, including TIP5, are parylated by PARP1. Recent progresses in PAR-mass spectrometry [163] will allow determining whether and how parylated NoRC complex affects the formation of rDNA heterochromatin. Alternatively, histone parylation might serve to destabilize nucleosomes to keep DNA more accessible to the action of DNA methyltransferases and/or of histone modifying enzymes. Moreover, parylation of histones might facilitate the deposition of silent histone modifications by docking chromatin enzymes.

The identification of PARP1 and parylation as regulators of rDNA silencing adds a further layer of complexity in the readout of PAR signalling. Notably, our assays did not detect formation of long PAR polymers, typically forming upon genotoxic signalling and generation of DNA strand breaks or in "puff" formation. If the length or the structure of the generated PARs might represent a critical mark that distinguishes PARP1 as coactivator or corepressor remains yet to be elucidated. Generation of antibodies specific to parylated histones and recent advances in PAR-mass spectrometry [163] will in the next future allow deciphering how the code of parylated histones or other chromatin and transcription regulators is mechanistically interpreted.

The ability of PARP1 and its enzymatic activity to establish silent rDNA chromatin and the association of PARP1 with the silent genes rRNA genes immediately after their duplication strongly suggest that PARP1 is one of the component of the machinery required to propagate silent marks at the rDNA locus (**Figure 8**). Future studies will address the mechanistic insights of how parylation of histones and of the NoRC complex mediates assembly of silent rDNA chromatin and whether similar mechanisms can take place at other constitutive heterochromatic regions (i.e. centromers) whose structure is required for genome stability. In conclusion, the data presented in this work unravel a novel function of PARP1 and ADP-ribosylation that serves to inherit silent chromatin structures, shedding light on how epigenetic marks are transmitted during each cell cycle.

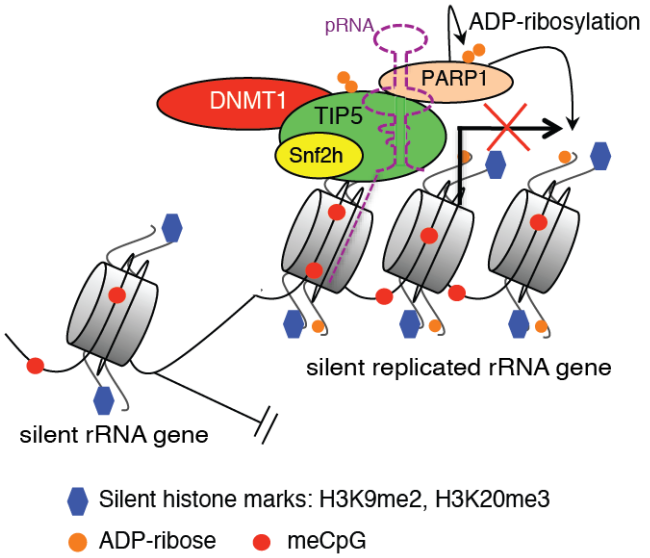


Figure 8 | Model showing the inheritance of silent rDNA chromatin mediated by TIP5, pRNA and PARP1 After the passage of the replication fork in mid S-phase, TIP5-pRNA binds to nascent rRNA genes. The pRNA region containing the loop structure mediates association of TIP5 and PARP1 and activates the enzymatic activity of PARP1 to parylate PARP1 itself, TIP5 or histones.

5 References

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Curriculum vitae

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PUBLICATIONS

Guetg C., Scheifele F., Rosenthal F., Hottiger M. O., and Santoro R., *Inheritance of silent rDNA chromatin is mediated by PARP1 via non-coding RNA.* Manuscript submitted for publication

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